

Virus Bioresistor (VBR) for Detection of Bladder Cancer Marker DJ-1 in Urine at 10 pM in One Minute

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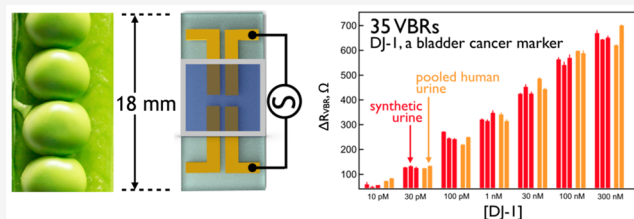


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ABSTRACT: DJ-1, a 20.7 kDa protein, is overexpressed in people who have bladder cancer (BC). Its elevated concentration in urine allows it to serve as a marker for BC. However, no biosensor for the detection of DJ-1 has been demonstrated. Here, we describe a virus bioresistor (VBR) capable of detecting DJ-1 in urine at a concentration of 10 pM in 1 min. The VBR consists of a pair of millimeter-scale gold electrodes that measure the electrical impedance of an ultrathin (≈ 150 – 200 nm), two-layer polymeric channel. The top layer of this channel (90–105 nm in thickness) consists of an electrodeposited virus-PEDOT (PEDOT is poly(3,4-ethylenedioxythiophene)) composite containing embedded M13 virus particles that are engineered to recognize and bind to the target protein of interest, DJ-1. The bottom layer consists of spin-coated PEDOT–PSS (poly(styrenesulfonate)). Together, these two layers constitute a current divider. We demonstrate here that reducing the thickness of the bottom PEDOT–PSS layer increases its resistance and concentrates the resistance drop of the channel in the top virus-PEDOT layer, thereby increasing the sensitivity of the VBR and enabling the detection of DJ-1. Large signal amplitudes coupled with the inherent simplicity of the VBR sensor design result in high signal-to-noise ($S/N > 100$) and excellent sensor-to-sensor reproducibility characterized by coefficients of variation in the range of 3–7% across the DJ-1 binding curve down to a concentration of 30 pM, near the 10 pM limit of detection (LOD), encompassing four orders of magnitude in concentration.



INTRODUCTION

Minimally invasive cancer screening using bodily fluids, so-called “liquid biopsies”, may eventually eliminate the evaluation of suspected malignancies using surgery.¹ Liquid biopsies involve the detection in blood, urine, and other bodily fluids of nucleic acids, circulating tumor cells (in blood), or distinctive protein markers that signal the presence of a particular cancer. DJ-1, a 20.7 kDa protein, is elevated in the urine of people with bladder cancer (BC).^{2,3} Presently, the measurement of DJ-1 in urine requires an enzyme-linked immunosorbent assay (ELISA), which is both slow and inconvenient. A biosensor for DJ-1 could accelerate its assessment for the detection of BC recurrence in patients who have undergone treatment for the disease. However, no biosensor for DJ-1 has been demonstrated to our knowledge. Here we demonstrate that a new type of biosensor, the virus bioresistor or VBR, that uses virus particles as receptors can be programmed to detect DJ-1 in human urine.

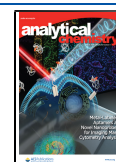
The VBR is a bioresistor contacted with two gold electrodes. The bioresistor consists of an electronically conductive channel composed of a layer of poly(3,4-ethylene dioxothiophene) (PEDOT) doped with virus particles. Recently,⁴ we demonstrated the VBR concept for the detection of human serum albumin (HSA, 66.5 kDa) in high salt (160 mM NaCl) buffer.

A limit-of-detection for HSA (LOD_{HSA}) of 7 nM was achieved in that study.⁴ However, a sub-1.0 nM LOD for protein markers is required to enable cancer surveillance in urine. Here we unlock higher sensitivity for VBRs simply by engineering the PEDOT channel to concentrate the impedance in an ultrathin (≈ 90 nm) virus-PEDOT composite layer. With this modification, a limit-of-detection of (LOD_{DJ1}) of 10 pM is achieved in urine (synthetic and human), coupled with a dynamic range of more than four orders of magnitude from 10 pM to 300 nM. This performance is clinically relevant because it allows for the detection of elevated DJ-1 in the urine of patients who have bladder cancer (≈ 100 pM).^{2,3,5,6} Importantly, the modifications to the VBR do not compromise either the speed or the simplicity of its operation. As before,⁴ the VBR operates in a dip-and-read modality and produces a stable, quantitative signal within 1.0 min. The sensing performance reported here also eclipses prior virus-based

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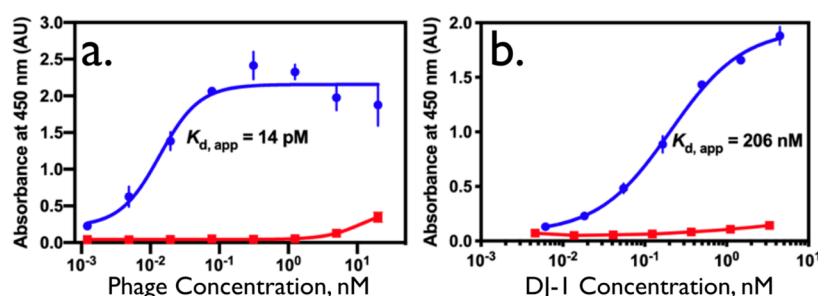


Figure 1. Two ELISAs for M13 phage binding of DJ-1. (a) Phage ELISA of the DJ-1-binding phages DL-1 (blue) and a negative control Stop-4 phages (red). Here, DJ-1 is immobilized, and the DL-1 phage is detected. The data were fit with a four-parameter logistic curve fit ($R^2 = 0.9230$). Measurements were performed in triplicate; error bars represent the standard deviation of the mean. (b) Sandwich ELISA of DJ-1. In this case, DL-1 phages (or the control Stop4 phage) are immobilized, and the DJ-1 protein is detected. This format mimics the function of the VBR. The data were fit as described above ($R^2 = 0.9944$). Measurements were performed in triplicate; error bars represent the standard deviation of the mean.

biosensors that we have studied over a period of 14 years in our laboratories.^{7–12}

The mechanism by which the VBR transduces protein binding remains under investigation. A hypothesis presented here proposes that a target protein permeates the virus-PEDOT layer as it undergoes affinity-driven partitioning to virus particles entrained in this layer. As the volume fraction of electrically insulating proteins increases, the electrical conductivity of the resistor channel imparted by PEDOT is reduced, generating the VBR signal.

EXPERIMENTAL METHODS

Materials and Methods. Gold electrodes were prepared by photolithography and physical vapor deposition. The following materials and reagents were purchased commercially and used as received: PMMA cells (Wainamics Inc., Fremont CA) and bare gold electrodes were oxygen plasma-cleaned (PDC-32G, Harrick Plasma). PEDOT-PSS (poly(3,4-ethylenedioxythiophene) polystyrenesulfonate) Heraeus Clevis PH1000 from Ossila; lithium perchlorate 99+% purity from Acros organics; EDOT (3,4-ethylenedioxythiophene) from Sigma-Aldrich; ethylene glycol from Macron Fine Chemicals. Phosphate-buffered saline (PBS, 10× concentrate) from Sigma-Aldrich. 1× concentrate of the PBS yielded a phosphate-buffered saline solution at pH 7.4 with a sodium chloride concentration of 0.154 M and a phosphate buffer concentration of 0.01 M. The DJ-1 overexpression plasmid pET3a-His-DJ1 was a gift from Michael J. Fox Foundation, MJFF (Addgene plasmid #51488). DJ-1 was recombinantly overexpressed in *E. coli*. Interleukin 6 (IL-6) was purchased from Tonbo Biosciences. M13 phage library design and procedures for the selection of DJ-1 binders using this library are described in the [Supporting Information](#) (SI).

The affinity of engineered M13 virus particles for DJ-1 can be seen from the results of two enzyme-linked immunosorbent assay (ELISA) measurements ([Figure 1](#)). The ELISA measurement was conducted two ways: with DJ-1 adsorbed onto a 96-well plate, measuring the recognition of adsorbed DJ-1 by phage particles in solution ([Figure 1a](#)), and by adsorbing the phage particles on the plate and measuring the binding of DJ-1 to this virus layer ([Figure 1b](#)). The latter configuration, which yields a much higher apparent dissociation constant, $K_{d,app}$ more closely resembles the operation of the VBR.

VBR Fabrication. The fabrication process for the VBRs is similar to that described previously⁴ with minor modifications. Briefly, gold-film electrodes were cleaned in an O₂ plasma for

10 min immediately before use. Thick (≈ 70 nm) and thin (≈ 48 nm) PEDOT-PSS films were prepared as follows: thick PEDOT-PSS films were obtained by stirring a solution of 3% (v/v) ethylene glycol with PEDOT-PSS for 30 min. Thin PEDOT-PSS films were obtained by stirring a solution of 1.5% (v/v) ethylene glycol in PEDOT-PSS for 30 min. These solutions were spin-coated on the gold electrodes at 2500 rpm for 80 s and then heated for 1 h at 90 °C. A PMMA cell was then attached to the PEDOT-PSS film, and PEDOT-PSS coated gold electrodes were equilibrated in PBS for 30 min. Next, virus-PEDOT films were electropolymerized onto the PEDOT-PSS/gold-film electrodes using a platinum foil counter and MSE reference electrodes. Virus-PEDOT films were then electrodeposited onto the PEDOT-PSS film from aqueous solutions containing 8 nM M13 bacteriophage, 12.5 mM LiClO₄, and 2.5 mM EDOT by performing two voltammetric scans from 0.2 to 0.8 V versus MSE at a scan rate of 20 mV/s. A PARSTAT 2263 controlled by Electrochemistry PowerSuite 2.6 software was used for this deposition. All VBRs employed for sensing measurements conformed to the screening parameters applied at each step of the fabrication process, as described in detail in the [SI](#). For additional detail, see [Figure S1](#).

Impedance Spectroscopy (IS). All solutions were prepared and equilibrated at room temperature (20 °C) prior to IS measurements. The VBR cell was first rinsed three times with PBS, after which impedance measurements were conducted as follows. Background IS measurement (in triplicate) was acquired in urine (synthetic or human) that contained no added protein, and a second IS measurement (again in triplicate) was acquired in the same urine containing added DJ-1 or IL-6 at the indicated concentrations. The difference in Z_{re} between these two measurements at each frequency is ΔR_{VBR} . The two R_{VBR} inputs to ΔR_{VBR} are obtained by fitting an impedance frequency spectrum that spans the range from 1 Hz to 40 kHz. All IS data were acquired using a Princeton Applied Research PARSTAT Model 2263 controlled by Electrochemistry PowerSuite 2.6 software. Fifty data points were acquired across a frequency range of 1 Hz to 40 kHz. The amplitude of the applied voltage was 10 mV for all IS measurements. VBRs are single use devices. A different VBR was therefore used for each measurement. Equivalent circuit fitting was accomplished using EIS Analyzer (ABC Chemistry). Minimization algorithm Powell (300 iterations) was used to generate values for each circuit element.

Time Scan Experiment. The time scan experiment was performed on four different VBRs for four concentrations of

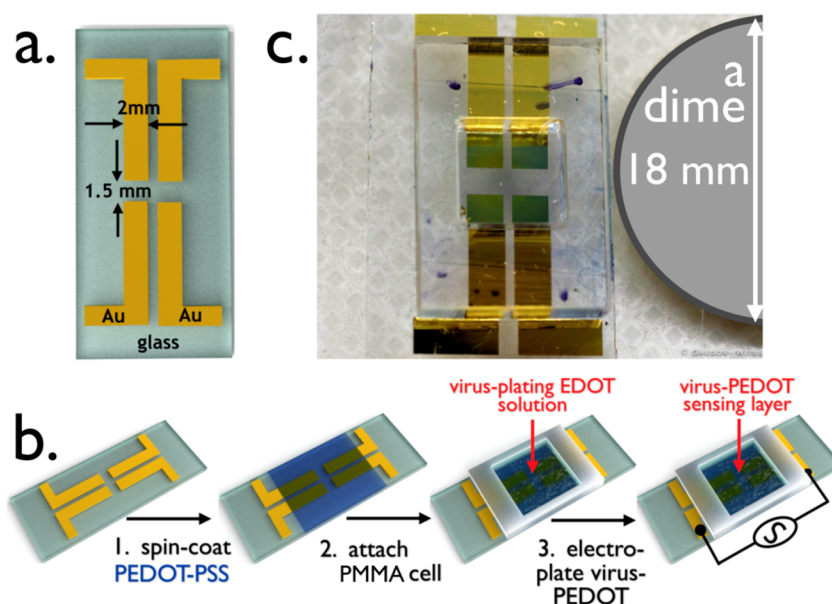


Figure 2. Virus bioresistor (VBR). (a) Rendering of gold electrodes for a two-VBR chip showing its dimensions. The two electrodes at left comprise one VBR and the two on the right a second VBR. These two VBRs will share a single bioaffinity layer. (b) Three-step process for fabricating a VBR: Step 1, a conductive PEDOT–PSS base layer is spin-coated onto the gold-on-glass template shown in panel a. This film is baked at 90 °C for 60 min; Step 2, a poly(methyl methacrylate)(PMMA) cell is attached on top of the dried PEDOT–PSS film; Step 3, the PMMA cell is filled with aqueous EDOT-virus plating solution, and a virus-PEDOT film is deposited by electrooxidation. This VBR biosensor is ready for use. (c) Photograph of a two-VBR chip with PMMA solution cell.

DJ-1 protein, 10 pM, 30 pM, 100 pM, and 1 nM. Each VBR was first equilibrated in synthetic urine for 9 min. A “pure” urine baseline R_{VBR} was then acquired at $f = 0.1$ Hz for 1.0 min. The synthetic urine was then removed from the PMMA cell and replaced with synthetic urine supplemented with DJ-1 protein at the specified concentration, without disconnecting the VBR from the potentiostat. After a 1 min exposure to the test solution, R_{VBR} was again recorded for 10 min.

Control Experiments. Three negative control experiments tested for signal specificity. In the first, a Stop-4 M13 virus, which has no displayed peptide binding moieties, was substituted for DJ-1-binding phage. The Stop-4 control VBRs were exposed to 500 nM DJ-1. Second, VBRs containing no phage were exposed to 500 nM DJ-1. Interleukin 6 (IL-6, 20.9 kDa, pI = 6.2), similar in size and pI to DJ-1, was used as a third control.

SEM Analysis. Scanning electron microscopy (SEM) data were acquired using a FEI Magellan 400L XHR FE-SEM. An accelerating voltage of 2 keV was used for uncoated films and 10 keV for samples coated with 3 nm of iridium.

RESULTS AND DISCUSSION

VBR Fabrication and Characterization. Starting with patterned gold electrodes on glass (Figure 2a), VBRs are prepared in three steps (Figure 2b). First, a PEDOT–PSS layer is deposited by spin-coating. Second, a poly(methyl methacrylate) or PMMA solution cell with adhesive backing is pressed onto the PEDOT–PSS layer. Third, this solution cell is used to electrodeposit a virus-PEDOT layer. This electrodeposition process applied the following protocol. The VBR cell is rinsed with PBS buffer and filled with an aqueous solution of EDOT (2.5 mM), LiClO_4 (12.5 mM), and engineered M13 virus particles (8 nM). Using a mercurous sulfate reference electrode (MSE), and a platinum counter electrode, the virus-PEDOT composite layer is electro-

deposited onto the PEDOT–PSS surface by scanning its potential (20 mV/s) from +0.20 V to +0.80 V and back versus MSE in two cycles (Figure 2a,b). Under these conditions, EDOT is oxidized, and the growth of EDOT oligomers proceeds until insoluble cationic PEDOT is precipitated as a film, together with charge-compensating ClO_4^- anions, onto the PEDOT–PSS electrode.¹³ If M13 virus is present in the plating solution, virus particles are incorporated into the growing film, a process promoted by the high negative charge density of these particles. At neutral pH, each M13 virion is blanketed with ~6000 negative charges.¹⁴ Previously, we have demonstrated that the electrodeposition of films from a plating solution containing M13 virus particles and EDOT produces a composite virus-PEDOT film that concentrates virus particles by a factor of 500 times relative to the M13 concentration in the plating solution.¹⁵ As seen in the photograph of a VBR shown in Figure 1c, the resulting VBR “channel”, consisting of a PEDOT–PSS bottom layer and a virus-PEDOT top layer, is transparent.

The VBR device architecture and polymeric channel resembles that of an organic electrochemical transistor (OECT).^{16–20} The differences between these two types of devices are the following. (1) The VBR is a two-terminal device with no gate electrode. This simplifies its operation considerably, as the VBR measures the impedance of its channel at its rest potential in the analysis solution without the need for gate scans and the requirement for optimization of the gate potential prior to a measurement.²¹ (2) The VBR measures an impedance frequency spectrum for the channel, typically across five orders of magnitude in frequency instead of the DC resistance of the channel, as is common practice with OECTs.^{22,23} This impedance data set allows the channel impedance, R_{VBR} , which provides the VBR signal, to be cleanly separated from the solution impedance, R_{soln} , which is correlated with the salt concentration of the analysis solution.⁴

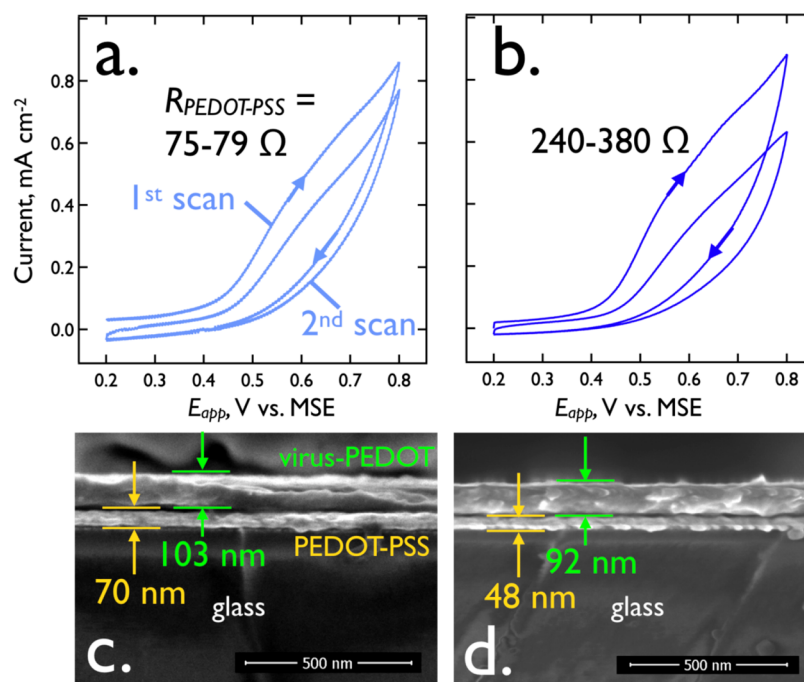


Figure 3. Electrodeposition and SEM cross sections of virus-PEDOT bioaffinity layers. (a) Virus-PEDOT bioaffinity layer is electrodeposited on a PEDOT-PSS base layer using two voltametric scans, as shown. The plating solution is aqueous 2.5 mM EDOT and 12.5 mM LiClO₄, 8 nM virus, and the scan rate is 20 mV/s. The DC resistance, $R_{\text{PEDOT-PSS}}$, of the PEDOT-PSS layer here is 75–79 Ω. (b) Same electrodeposition process for a thinner, PEDOT-PSS base layer with $R_{\text{PEDOT-PSS}}$ in the range from 240–380 Ω. (c, d). Cross-sectional SEM images of these two layers show that the more conductive PEDOT-PSS layer ($R_{\text{PEDOT-PSS}}$ = 75–79 Ω) is 70 nm (± 3 nm) in thickness, whereas the less conductive PEDOT-PSS layer is 48 nm (± 2 nm) in thickness. The electrodeposited virus-PEDOT layer is also somewhat thinner in panel d relative to panel c in accordance with the lower deposition currents observed for the second deposition scan.

For bodily fluids such as urine, R_{soln} has the potential to provide information relating to the hydration state of a patient. (3) VBRs use engineered virus particles as receptors. Virus particles may be entrained in a PEDOT film by coelectrodeposition of the virus with the polymer as described above. A fourth difference may be the mechanism of signal generation, as described below.

The architecture and resultant properties of the VBR channel dictate its sensing performance. We focus attention here on the importance of the PEDOT-PSS layer thickness and electrical resistance. SEM cross-sectional images (Figure 3c,d) show that both polymer layers are tens of nanometers in total thickness. The thickness of the PEDOT-PSS bottom layer is influenced both by the presence of ethylene glycol (EG) in the deposition solution^{24,25} and the spin coater speed. The addition of EG is known to increase the conductivity of PEDOT-PSS by altering its morphology.^{26,27} Relatively thick (70 (± 3) nm) low resistance films were obtained using 3% (v/v) EG, while high resistance films (48 (± 2) nm) were prepared using 1.5% (v/v) EG (Figure 3c,d). The electrical resistance of these layers, $R_{\text{PEDOT-PSS}}$, is = 75–79 Ω (thick) and 240–380 Ω (thin). The increased resistance of the PEDOT-PSS bottom layer has little effect on the thickness of the virus-PEDOT top layer electrodeposited on it, and the virus-PEDOT top layers have similar thicknesses of 92 (± 4) nm (high PEDOT-PSS resistance) versus 103 (± 4) nm (low resistance). As we demonstrate below, a reduction in thickness of the PEDOT-PSS layer, and an increase in its resistance, boosts the sensitivity of the VBR for the detection of HSA and DJ-1.

Electrodeposited virus-PEDOT and PEDOT-only films have a characteristic topography imparted by PEDOT crystallites protruding by up to a micron from the planar surface of the

PEDOT film (Figure 4). These “PEDOT stalagmites” are not related to virus particles as they are observed both in the absence (Figure 4a,b) and presence (Figure 4c,d) of added phage particles. PEDOT stalagmites have attributes of crystallites including a faceted appearance, as previously reported in the literature.^{28,29} In virus-PEDOT films, entrained M13 virus particles appear as black filamentous objects against a gray PEDOT background (Figure 4c,d,e). SEM examination of several samples shows that the virus concentration within the plane of the virus-PEDOT film is nonuniform with 10–30 μm^2 regions that are intensely black, indicating high virus concentrations, and other regions that are gray with a relatively low virus concentration. The clustering of virus particles within the film is interesting and surprising, given the high negative charge density of these particles.

VBR Electrical Response and Signal. As previously proposed,⁴ a simple equivalent circuit containing four circuit elements accounts for the measured frequency-dependent impedance of the VBR channel from DC to 40 kHz (Figure 5a). In this circuit, the capacitance of the virus-PEDOT/solution interface is represented by a total capacitance, C . This capacitance provides coupling between the AC voltage signal applied to the channel and the analyte solution. Three resistors represent the resistance of the analyte solution (R_{soln}), the resistance of the top polymer layer ($R_{\text{PEDOT-virus}}$), and the resistance of the bottom PEDOT-PSS layer ($R_{\text{PEDOT-PSS}}$).

The impedance response of a VBR is characterized by a semicircular Nyquist plot (Z_{im} versus Z_{re} , Figure 5b). A qualitative understanding of the VBR response is provided by examining its limiting behaviors at low and high frequencies across the range from 1.0 Hz to 40 kHz. At $f = 1.0$ Hz, the capacitive reactance of the virus-PEDOT/solution interface,

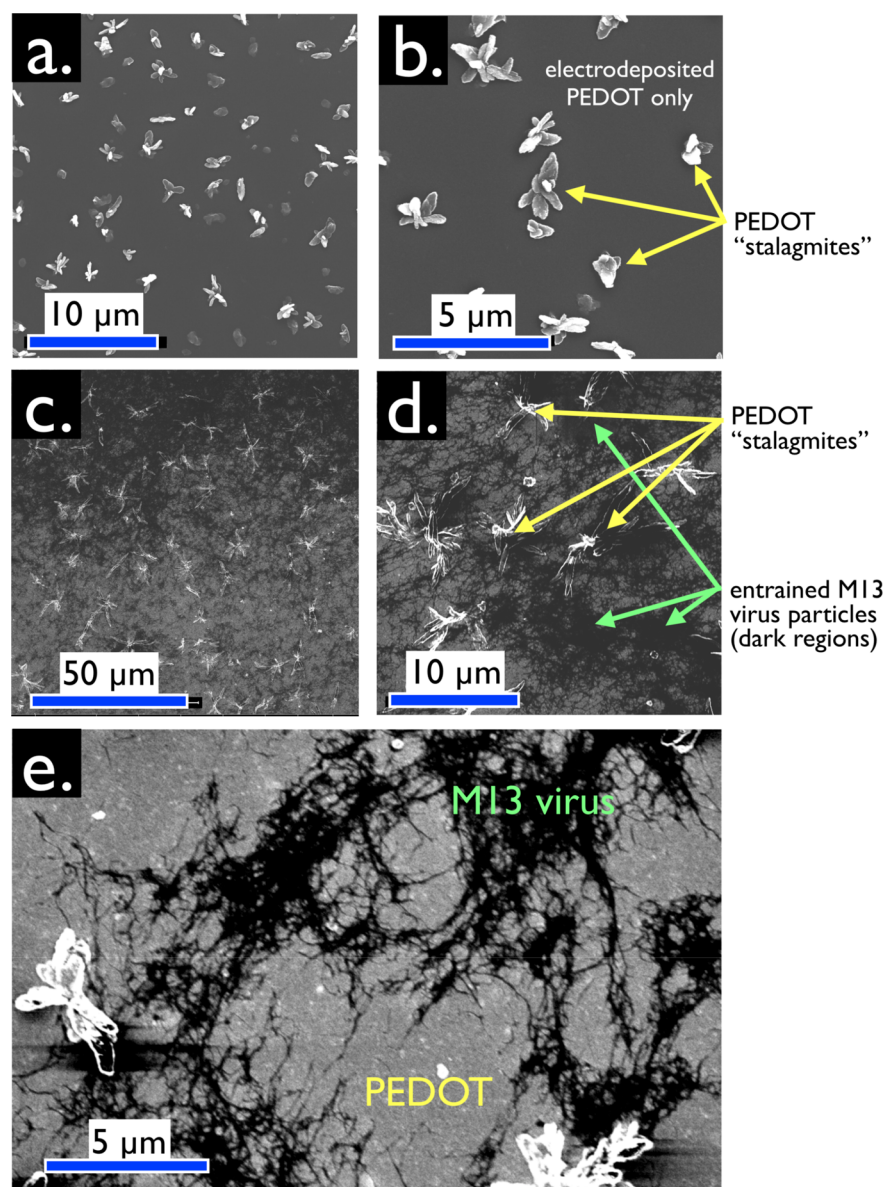


Figure 4. Plan-view SEM images, acquired with secondary electron detection (SED), of (a, b) virus-free and (c, d, e) virus-containing bioaffinity layers. (a, b) Control VBR bioaffinity layer prepared by electrodeposition from a solution containing no virus particles. Micron scale protrusions from the surface of this film are characteristic of electrodeposited PEDOT. These protrusions are not seen at PEDOT–PSS films prepared by spin-coating. We refer to these structures as “PEDOT stalagmites”. (c, d, e) VBR bioaffinity layers containing M13 virus particles. Filamentous M13 virus particles comprise the dark regions of these images. Lighter gray regions contain no virus. PEDOT stalagmites are also observed. (e) Enhanced contrast exposes tangles of M13, again distributed nonuniformly inside a virus–PEDOT bioaffinity layer.

($Z_C = (2\pi fC)^{-1} \approx 6 \text{ k}\Omega$) is larger than R_{VBR} ($\approx 2.1\text{--}2.7 \text{ k}\Omega$, Table 1). Therefore, although R_{soln} is small by comparison to R_{VBR} ($289\text{--}330 \Omega$), the value of Z_C strongly attenuates the AC signal that accesses R_{soln} . In this limit, R_{VBR} is approximated by the parallel combination of $R_{PEDOT-virus}$ and $R_{PEDOT-PSS}$ (R_{VBR} , eq 1):

$$R_{VBR} \approx \frac{(R_{PEDOT-PSS})(R_{PEDOT-virus})}{R_{PEDOT-PSS} + R_{PEDOT-virus}} \quad (1)$$

As shown below and previously,² R_{VBR} increases in the presence of a target protein that is bound by virus particles in the virus–PEDOT layer. The difference between R_{VBR} in the presence and absence of this protein is the VBR signal, ΔR_{VBR} .

At the high frequency limit, $f = 40 \text{ kHz}$, the capacitive reactance approaches zero ($Z_C = (2\pi fC)^{-1} \approx 0.15 \Omega$), and the circuit of Figure 5a simplifies to three resistors in parallel:

$$R_{VBR} \approx \frac{(R_{PEDOT-PSS})(R_{PEDOT-virus})(R_{soln})}{R_{PEDOT-PSS}R_{PEDOT-virus} + R_{soln}R_{PEDOT-virus} + R_{soln}R_{PEDOT-PSS}} \quad (2)$$

At $f = 40 \text{ kHz}$, R_{VBR} is much lower than at 1 Hz because the small resistor R_{soln} is accessed in parallel to $R_{PEDOT-PSS}$ and $R_{virus-PEDOT}$. To a first approximation, the impedance at both of these frequency limits, 1.0 Hz and 40 kHz, is purely resistive but at intermediate frequencies, a significant capacitive component is introduced, producing the characteristic semi-circular Nyquist plot that is observed, as seen in Figure 5b.

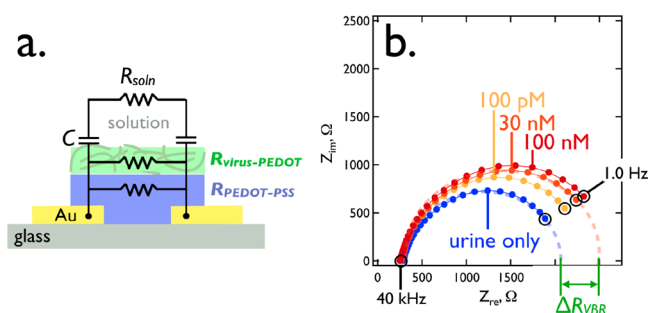


Figure 5. (a) VBR equivalent circuit and a (b) typical impedance response. This Nyquist plot (Z_{im} versus Z_{re}) shows the impedance frequency spectrum plotted between 1.0 Hz and 40 kHz for a synthetic urine solution that was supplemented with DJ-1. A single VBR measured these solutions at the indicated DJ-1 concentrations.

The values of C , R_{soln} , and R_{VBR} (encompassing $R_{PEDOT-virus}$ and $R_{PEDOT-PSS}$) are obtained by deconvolution of the complex impedance data set. How do R_{VBR} , R_{soln} , and C change in response to the concentration of a target protein? For DJ-1 concentrations from 0–100 nM, variations of R_{soln} are constant within the error bars for this measurement and are independent of DJ-1 concentration (Table 1). The capacitance, C , approximated as a constant phase element (CPE, $Z_C \approx Z_{CPE} = -\frac{1}{Q(i\omega)^n}$), varies weakly with the DJ-1 concentration (Table 1). R_{VBR} at low frequency, in contrast, is strongly correlated with the DJ-1 concentration and, as already indicated, ΔR_{VBR} is used to transduce the concentration of a target protein bound by entrained virus particles (Table 1).

Since, as noted above, the VBR signal ΔR_{VBR} is best measured at low frequency, would it not be simpler to use the DC resistance of the VBR channel to derive signal? In practice, the DC measurement does work, but there are two reasons for measuring the frequency spectrum instead. (1) In DC sensing mode, one does not acquire the high frequency impedance (eq 2) that permits deconvolution (and measurement) of the solution resistance from the ΔR_{VBR} signal. (2) The reproducibility of the impedance at low frequency is better than for a DC measurement. That is, the signal-to-noise at low frequencies down to 1 Hz is higher than the noise at DC (data not shown).

Tuning VBR Signal Amplitude Using $R_{PEDOT-PSS}$. Eq 1 predicts that at low frequencies, increasing $R_{PEDOT-PSS}$ causes R_{VBR} to converge on $R_{PEDOT-virus}$ (Figure 6a). If ΔR_{VBR} is generated by the virus-PEDOT top-layer, then an increase in $R_{PEDOT-PSS}$ should increase VBR sensitivity. This expectation is confirmed by measurement of ΔR_{VBR} for the protein human serum albumin, HSA, a 66.5 kDa protein that is a marker for

renal failure (Figure 6). A plot of ΔR_{VBR} versus $R_{PEDOT-PSS}$ for [HSA] = 100 nM shows that increasing $R_{PEDOT-PSS}$ is from 70 Ω to 380 Ω by reducing thickness of this layer increases the ΔR_{VBR} from 40 Ω to more than 500 Ω (Figure 6b).

Nyquist plots for three $R_{PEDOT-PSS}$ values (Figure 6c,d,e) document the increase in sensitivity for three VBRs. It should be noted that $R_{PEDOT-PSS} \approx 300 \Omega$ is a practical upper limit in our experiments. Attempts to further thin the PEDOT-PSS layer to achieve even higher sensitivities resulted in pronounced irreproducibility in both $R_{PEDOT-PSS}$ and measured ΔR_{VBR} values.

Two calibration plots for HSA in PBS buffer solution acquired using VBRs compare the performance of high resistance PEDOT-PSS layers ($R_{PEDOT-PSS} = 260$ – 300Ω) with low resistance PEDOT-PSS layers ($R_{PEDOT-PSS} = 80$ – 100Ω , Figure 6f). The ΔR_{VBR} signal for HSA increases by between 10 $\times$ (at low concentrations) to 3 $\times$ (at high concentrations) across the HSA concentration range encompassed by these data.

$R_{PEDOT-PSS}$ tuning of the VBR sensitivity also works for DJ-1—a bladder cancer marker that is significantly smaller than HSA (20.7 kDa versus 66.5 kDa). Again, a plot of ΔR_{VBR} versus $R_{PEDOT-PSS}$ for a concentration of DJ-1 of 100 nM in synthetic urine shows that increasing $R_{PEDOT-PSS}$ from 75 Ω to 300 Ω increases ΔR_{VBR} from 50 Ω to 550 Ω (Figure 7a). Nyquist plots for three $R_{PEDOT-PSS}$ values (Figure 7b,c,d) document the increase in sensitivity for three VBRs.

In looking more carefully at the DJ-1 sensing performance of VBRs with high resistance PEDOT-PSS layers ($R_{PEDOT-PSS} \approx 300 \Omega$), Nyquist plots (Figure 8a–c) show the accessible DJ-1 dynamic range extends from a limit-of-detection of 10 pM to 300 nM, a range of more than four orders of magnitude (see values of all circuit elements for these three VBR sensors in Table S1). A plot of ΔR_{VBR} versus DJ-1 concentration across this same range for a total of 35 VBR sensors (Figure 8d) conforms to the Hill equation:³⁰

$$\Delta R_{VBR} = \Delta R_{VBR,0} + \frac{\Delta R_{VBR,lim} - \Delta R_{VBR,0}}{1 + \left(\frac{K_D}{[DJ-1]}\right)^h} \quad (3)$$

A best fit of eq 3 to these data yields the following parameter values: $\Delta R_{VBR,lim} = 950 \pm 640 \Omega$, $\Delta R_{VBR,0} = 50 \pm 140 \Omega$, $K_D = 39 \pm 170$ nM, $h = 0.3 \pm 0.2$, and $R^2 = 0.94$. It should be noted that these data encompass measurements of DJ-1 in synthetic urine (21 sensors) and in pooled human urine (14 sensors). Each of these VBRs was used for a single DJ-1 concentration data point in Figure 8d. The measured value of h indicates strong negative cooperativity, meaning that the microscopic dissociation constant, K_D , is increased (the affinity interaction

Table 1. VBR Circuit Element Values, and ΔR_{VBR} Corresponding to the Nyquist Plots of Figure 5b

	synthetic urine		100 pM DJ-1		30 nM DJ-1		100 nM DJ-1	
	value	st dev	value	st dev	value	st dev	value	st dev
R_{soln} (Ω)	331	8	288.7	0.9	293	7	279.3	0.2
R_{VBR} (Ω)	2156	1	2436	5	2641	4	2733	8
ΔR_{VBR} (Ω)	0		280		485		577	
CPE, Q (F) ^a	2.77×10^{-5}	0.03×10^{-5}	2.59×10^{-5}	0.01×10^{-5}	2.65×10^{-5}	0.02×10^{-5}	2.520×10^{-5}	0.009×10^{-5}
CPE, n ^a	0.84	0.00	0.85	0.00	0.85	0.00	0.86	0.00

^aConstant phase element (CPE) approximation of the capacitance, C .⁵² $bZ_{CPE} = -\frac{1}{Q(i\omega)^n}$. The phase angle, θ , of the impedance response is, $\theta = -(90n)^\circ$, with $0 > n > 1$. $n = 1$ corresponds to ideal capacitor behavior.

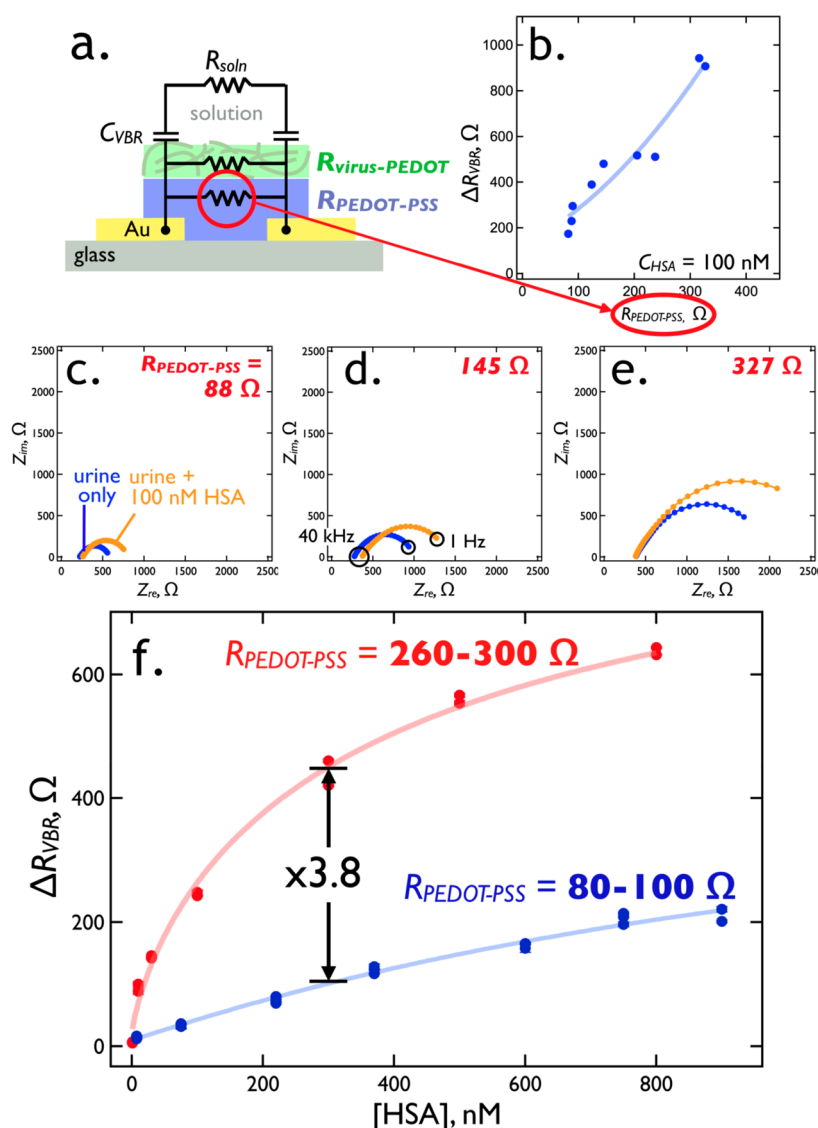


Figure 6. $R_{\text{PEDOT-PSS}}$ tuning of the VBR sensitivity for HSA. (a) Equivalent circuit for the VBR places the electrical impedance of the virus-PEDOT layer, R_{VBR} , in parallel with that of the PEDOT-PSS bottom layer, $R_{\text{PEDOT-PSS}}$, forming a current divider. (b) Increasing $R_{\text{PEDOT-PSS}}$ from 80 Ω to 300 Ω , by reducing the PEDOT-PSS layer thickness, forces current, i , through the virus-PEDOT measurement layer, increasing the signal for 100 nM HSA by a factor of 3 to 5 from 200 Ω to more than 900 Ω . (c, d, e) Three Nyquist plots corresponding to three values of the resistor, $R_{\text{PEDOT-PSS}}$, as indicated. In each plot, impedances are plotted in the complex plane from 1 Hz (right) to 40 kHz (left). A shift in the low frequency Z_{re} from synthetic urine only (blue trace) to 100 nM DJ-1 (orange trace) approximates the signal, ΔR_{VBR} . (f) R_{VBR} versus [HSA] calibration plots for a series of 42 VBR sensors (21 in each plot) with $R_{\text{PEDOT-PSS}}$ values in the range from 80 to 100 Ω and 260 to 300 Ω . The higher $R_{\text{PEDOT-PSS}}$ devices produce 3–5-times more signal amplitude across the HSA binding curve.

is reduced) as the fraction of binding sites occupied by the target protein increases.³⁰ This has the effect of stretching the binding curve across a wider range of DJ-1 concentration range, exceeding four orders of magnitude in the present case (Figure 8d).

In principle, the absence of a gate and an applied gate potential referenced to an external reference electrode leaves open the possibility of potential drift of the channel that could drive doping and dedoping reactions of the PEDOT sensing layers, causing baseline drift of the sensor and degrading reproducibility of the concentration measurements. However, the data of Figure 8e show that this “channel conductivity drift” is inconsequential on the time scale of 1–2 min required for carrying out VBR measurements of concentration. To this end, we made measurements of DJ-1 conducted in triplicate (for synthetic urine) and duplicate (for human urine)

document the reproducibility of VBRs (Figure 8e). Sensor-to-sensor coefficients-of-variation (CoV) vary from 2.9% (30 pM), to 4.1% (300 nM), extraordinarily low values. As expected, at the 10 pM LOD, a higher CoV of 19% is obtained. It should be noted that VBRs are effectively single use devices because the off-rate for bound DJ-1 after a single exposure is several hours (data not shown). This means that individual VBRs cannot be calibrated; every VBR sensor must respond to the same calibration curve placing a premium on the sensor-to-sensor reproducibility.

Nonspecific adsorption at the unmodified virus-PEDOT surface of a VBR is negligible, contributing to the simplicity of VBR fabrication (Figure 2b). Blocking, often accomplished by pre-equilibrating a bioaffinity layer with solutions of bovine serum albumin (BSA), casein,^{31–33} or poly (ethylene glycol),³⁴ prior to exposure to a target protein, is not required. Three sets

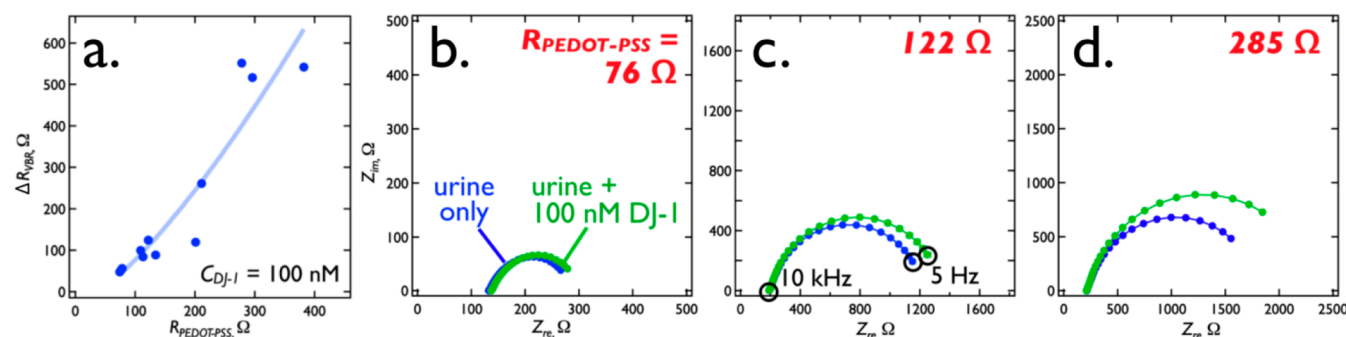


Figure 7. $R_{PEDOT-PSS}$ tuning of the VBR sensitivity for DJ-1. (a) Increasing $R_{PEDOT-PSS}$ from 80 Ω to 300 Ω by reducing the PEDOT-PSS layer thickness increases the signal for 100 nM DJ-1 by a factor of ≈ 10 from 50 Ω to 550 Ω . (b, c, d) Three Nyquist plots corresponding to three values of the resistor, $R_{PEDOT-PSS}$, as indicated. In each plot, impedances are plotted in the complex plane from 1 Hz (right) to 40 kHz (left). A shift in the low frequency Z_{re} from synthetic urine only (blue trace) to 100 nM DJ-1 (green trace) approximates the signal, ΔR_{VBR} .

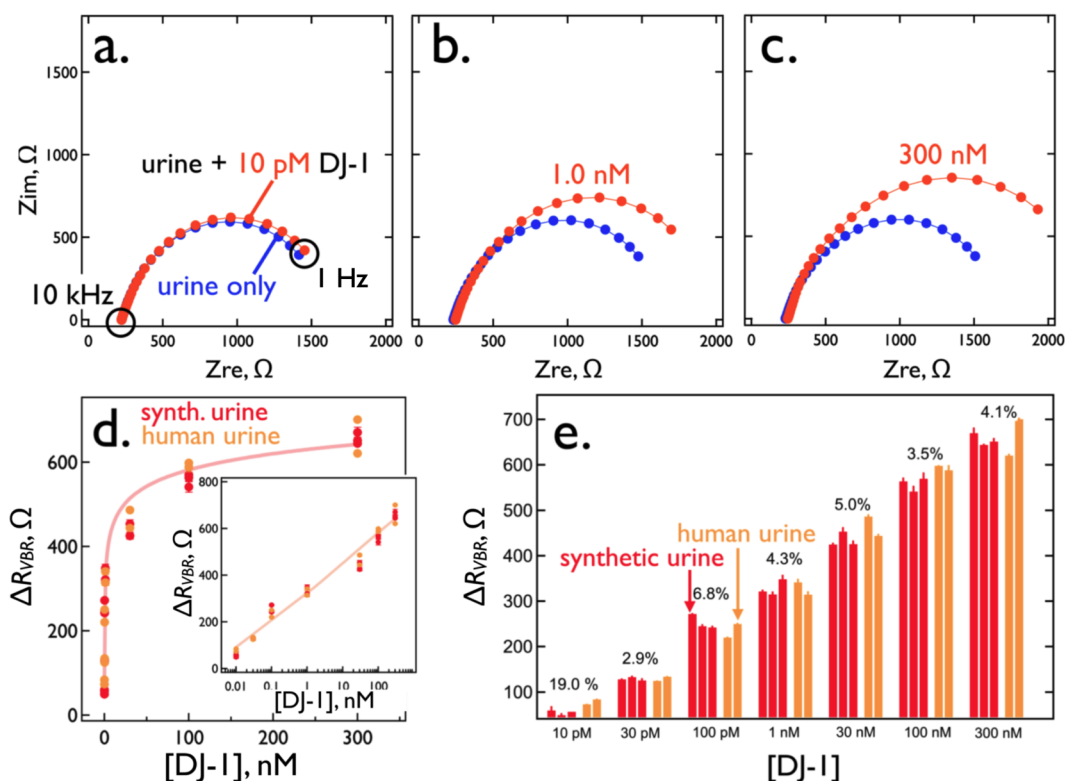


Figure 8. DJ-1 sensing performance using VBRs with $R_{PEDOT-PSS} = 280$ to 300Ω . (a, b, c) Nyquist plots for three DJ-1 concentrations of (a) 10 pM, (b) 1 nM, and (c) 300 nM. Also shown (blue traces) are background Nyquist responses in synthetic urine only. (d) Calibration curve for the detection of DJ-1 using the R_{VBR} signal constructed using 21 individual measurements from the same number of VBRs, at seven concentrations. Values of K_D and h (the Hill exponent) obtained from a best fit of the experimental data to eq 2 are indicated. (e) Bar plot for ΔR_{VBR} measurements acquired from 21 electrodes, illustrating the sensor-to-sensor reproducibility of these data. CoVs for these data, shown, are in the 2 to 8% range across 4 orders of magnitude in DJ-1 concentration. The values of each of the circuit elements is indicated in Table S1.

of negative controls (Figure 9a) for VBRs containing no phage, VBRs prepared using Stop-4 phage (which has no displayed peptides at its surface), and VBRs prepared using DJ-1 binding phage in the presence of interleukin 6 or IL-6 (a protein of similar size, 20.9 kDa, and pI, 6.2 versus 6.7 for DJ-1) either produce no measurable signal or a small “negative” signal, corresponding to a negative value of ΔR_{VBR} (Figure 9a).

Analysis speed and simplicity of operation are two requirements for biosensors that are used either at the point-of-care (PoC) or outside a care facility, at a point-of-need (PoN).^{35,36} The VBR provides for detection of DJ-1 across a range of concentrations within one minute in a dip-and-read

modality (Figure 9b). Thus, the VBR is well-adapted to PoC and PoN applications.

The frequency-dependent signal-to-noise ratio (S/N) for the VBR (Figure 10a) increases with decreasing frequency from 40 kHz to 1.0 Hz. In this measurement, noise is defined as the standard deviation of repetitive measurements ($N = 3$) for ΔR_{VBR} (the signal) at a defined concentration of target protein. At a DJ-1 concentration of 300 nM, S/N peaks at 150 at 1.0 Hz and decreases to 60 at 100 pM and 4 at 10 pM, the limit-of-detection. S/N ratios at 40 kHz, in contrast, are in the range from 2 to 14 for this range of DJ-1 concentration. Consistent with the equivalent circuit of Figure 5a, increasing frequency

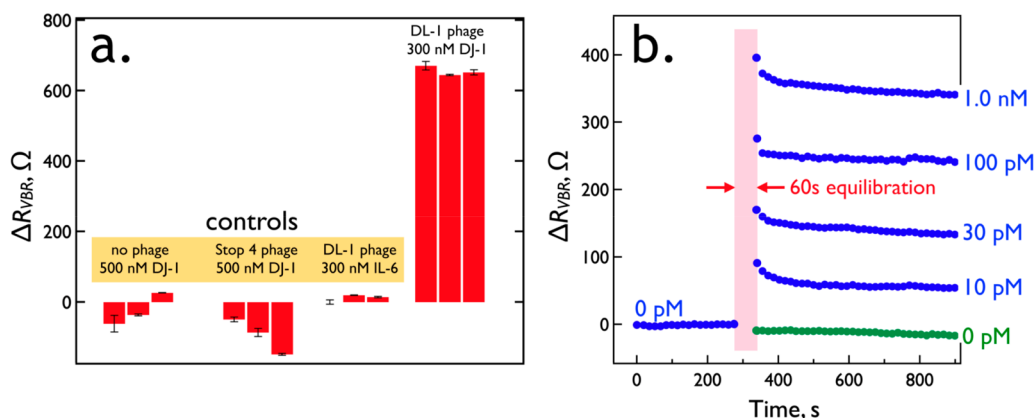


Figure 9. VBR specificity and speed. (a) Three control experiments. At left is the response of three VBRs prepared with no phage exposed to 500 nM DJ-1. To the right of this is the response of three VBRs prepared with Stop-4 phage that has no displayed peptides on its surface. Finally, at right are shown the results of three VBRs containing DL1 phage (selected for the binding to DJ-1) upon exposure to IL-6, a protein of similar MW (20.9 kDa) and pI (6.2) to DJ-1 (20.7 kDa and pI of 6.7, respectively). (b) Real-time VBR sensing data. Responses for five VBR sensors are shown for DJ-1 exposures of 0 pM (green trace), 10 pM, 30 pM, 100 pM, and 1.0 nM. These traces were obtained by first stabilizing sensors in synthetic urine for 9 min, measuring a R_{VBR} baseline at 0.10 Hz, and then interrupting for 1.0 min while the synthetic urine was replaced with synthetic urine supplemented with DJ-1 at the specified concentration, after which ΔR_{VBR} signal was acquired.

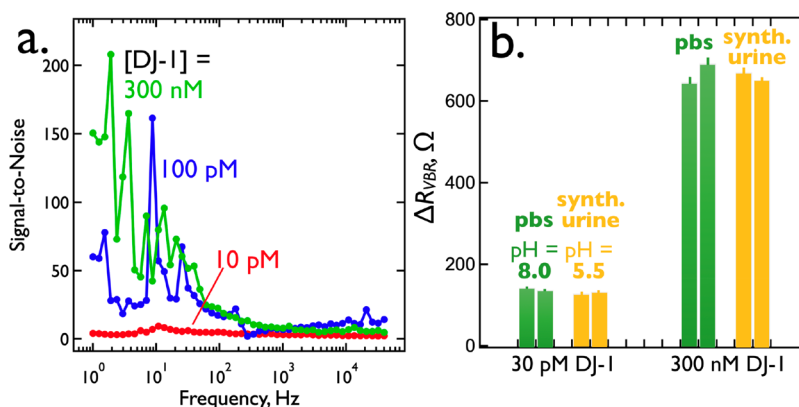


Figure 10. (a) Signal-to-noise (S/N) versus frequency for the detection of DJ-1. Shown are three plots of S/N versus frequency measured for three VBRs immersed in three DJ-1-spiked synthetic urine solutions containing DJ-1 at the indicated concentrations. Noise is calculated as the standard deviation of three replicate measurements at each frequency. S/N consistently increases from high to low frequencies. (b) Comparison of ΔR_{VBR} for the detection of DJ-1 in two electrolytes: synthetic urine (pH = 5.5) and PBS buffer (pH = 8.0). The charge state of DJ-1 (pI = 6.7) inverts across this pH difference, and is negatively charged at pH = 8.0 and positively charged at pH = 5.5. However, ΔR_{VBR} at two different concentrations are the same, despite changes in pH, within the reproducibility of these measurements.

reduces the impedance of the virus-PEDOT solution capacitance, Z_C , opening a low impedance path through the analyte solution, R_{soln} , and bypassing the signal-generating current path of the channel. It should be noted that the S/N versus frequency data sets (Figure 10a) are themselves noisy. Repeated measurements of these data show that the sharp peaks and valleys seen in these traces are not reproduced. This means that there are temporal variations in the noise present in the VBR circuit. However, the trend of increasing S/N with decreasing frequency remains prominent in these data.

Proposed Mechanism for VBR Signal Generation. The mechanism by which the VBR produces an impedance increase in the presence of target protein is of interest. This mechanism must account for three experimental observations: (1) ΔR_{VBR} is positive. In particular, the sign of the protein charge, positive or negative, has no influence on the signal. For example, DJ-1 (pI = 6.7) can be measured both at pH = 5.5 and at 8.0, corresponding to a positively charged protein, and a negatively charged protein, respectively (Figure 10b). ΔR_{VBR} signal is unaffected by this charge inversion. (2) VBR signal is

insensitive to the salt concentration of the test solution. Previously,⁴ we demonstrated this for the detection of 75 nM HSA in salt solutions ranging from 134 mM to 670 mM NaCl where no significant change in HSA signal was observed. Collectively, (1) and (2) imply that a charge gating mechanism, responsible for signal in field-effect transistors,³⁷ cannot be operating in VBRs. (3) The signal-to-noise ratio is strongly frequency dependent: S/N is high at low frequency (≈ 1 Hz) and near zero at high frequency (≈ 40 kHz) where it is also independent of the concentration of a target protein. The implication is that the signal generating process does not require the transmission of AC signal through electrolyte. This includes electrolyte that is present in the voids within the porous virus-PEDOT signal-generating layer. Thus, R_{VBR} decreases by just 24 Ω or 4% (against a background of 600 Ω) in protein-free aqueous NaCl as C_{NaCl} is increased by a factor of 50 from 0.02 to 1.00 M (Figure S2).

A simple mechanism that may account for these observations is shown schematically in Figure 11. Here, just the virus-PEDOT layer is illustrated. The virus-PEDOT layer

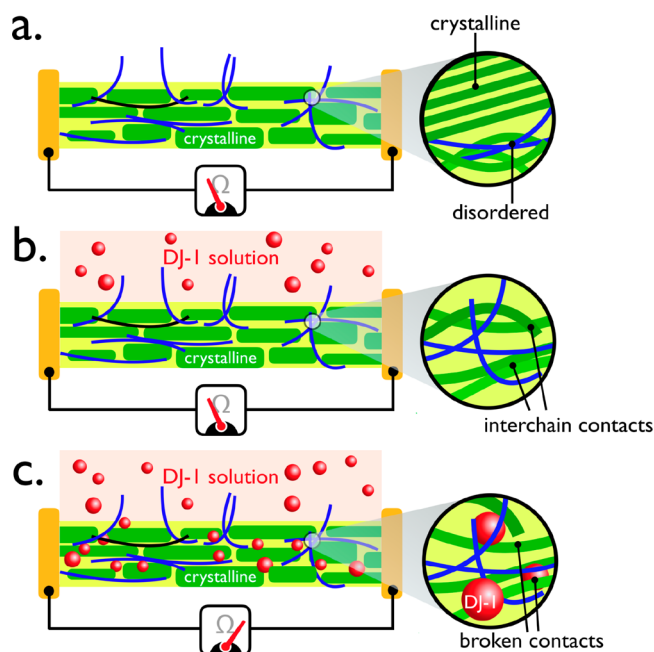


Figure 11. Schematic representation of a hypothesized signal transduction mechanism for the VBR. (a) The virus-PEDOT layer shown here consists of semicrystalline PEDOT with virus particles that are concentrated within disordered regions of the PEDOT layer. (b) When exposed to the DJ-1 protein solution, ΔR_{VBR} is initially zero, because an induction time is associated with the rate-limiting diffusion of the protein into the virus-PEDOT layer. (c) Permeation of the virus-PEDOT layer by DJ-1 is associated with an increase in its resistance as the insulating protein interferes with conduction pathways within this layer.

itself is semicrystalline, containing crystalline PEDOT-only domains surrounded by disordered domains that contain

disordered PEDOT chains and, likely, most of the virus particles (Figure 11a). Electrical conduction within this layer occurs by two processes: (i) intrachain charge transport (via bipolarons) and (ii) interchain electron hopping (Figure 11a). Interchain hopping, in particular, can be disrupted by the partitioning of protein into this layer, promoted by the immunoaffinity partitioning of target protein by virus particles entrained in the virus-PEDOT film.³⁸

This mechanism is analogous to that proposed for chemiresistive gas sensors that exploit a carbon/polymer composite chemiresistor.^{39–43} In these systems, permeation of a carbon/polymer (insulating) composite by a molecule in the vapor phase causes an increase in the volume of the composite and a decrease in the volume fraction of the conducting carbon phase, leading to a reduction in the conductivity of the composite, a process that is described by percolation theory:^{44,45}

$$\sigma = \sigma_0 [V - V_c]^\alpha \quad (4)$$

where σ_0 is the conductivity of the composite in the absence of permeating vapor species, σ is the conductivity of the composite after exposure to this vapor, V is the volume fraction of the conductive component of the composite, V_c is the volume fraction of the conductive phase at the percolation threshold, and α is a scaling exponent that depends only on the dimensionality of the percolation process (2-dimensional or 3-dimensional). Eq 4 is intended to model the conductivity at values of V near the percolation threshold, but more generally, it provides a signal transduction mechanism for chemiresistors in which the resistor is composed of a nonconductive and a conductive component and for which permeation of analyte(s) induces swelling of this system.

The mechanism depicted (Figure 11) requires that target protein diffuses into the virus-PEDOT layer. Does this occur

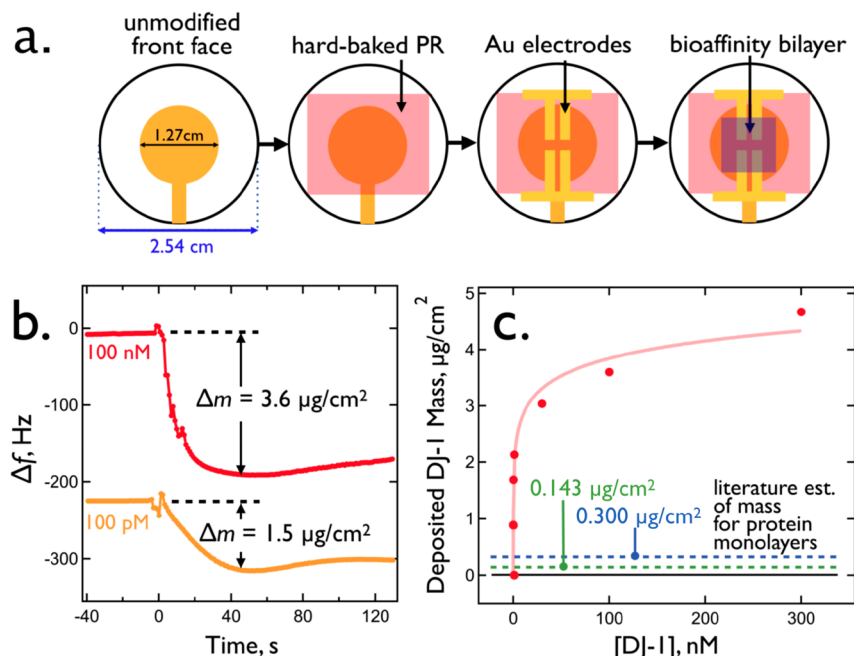


Figure 12. Measurement of DJ-1 mass loading for VBR bioaffinity layers using quartz crystal microbalance (QCM) gravimetry. (a) Four step fabrication of a complete VBR bioaffinity layer on a commercial QCM crystal. (b) Change in frequency, Δf , versus time for the exposure of two VBRs to solutions of DJ-1 at 100 pM and 100 nM. The measured DJ-1 mass loading is indicated and traces are shifted along the Δf axis for clarity. (c) Concentration of DJ-1 versus its measured mass loading. This isotherm was acquired in PBS buffer solution.

on the one-minute time-scale of signal generation, and if so, what mass loading of protein is obtained in this layer during this brief period? These questions can be addressed using quartz crystal microbalance (QCM) gravimetry. In this measurement, a two-layer PEDOT–PSS + virus-PEDOT bioaffinity layer is prepared on a QCM crystal coated with a thin photoresist layer. Exactly the same processes used for VBR fabrication (Figure 12a), involving the PVD deposition of gold electrodes, spin-coating of the PEDOT–PSS layer, and electrodeposition of the virus-PEDOT layer, are used for the preparation of these layers; for these experiments, the gold electrodes are not used to make electrical measurements.

Upon exposure to DJ-1 solutions, the resonant frequency, f_R , decreases within seconds and stabilizes within ~ 40 – 60 s (Figure 12b). Using the Sauerbrey equation,⁴⁶ the observed frequency change, Δf , can be translated into a mass change, Δm :

$$\Delta f = -\frac{f_R}{\rho_q d A} \Delta m \quad (5)$$

where f_R is the resonant frequency of the quartz crystal oscillator in air, ρ_q is the density of this crystal, d is its thickness, and A is the area of the gold electrodes deposited onto this crystal.

A plot of Δm versus DJ-1 concentration shows saturation behavior resembling the VBR calibration plot for DJ-1 (Figure 8d). The mass loading saturates at ~ 4 $\mu\text{g}/\text{cm}^2$, which corresponds to more than 1.0 monolayer of DJ-1 at the surface of the virus-PEDOT layer, even if this layer has significant roughness. For purposes of comparison, the mass of a hydrated protein monolayer has been measured using QCM for several proteins including RNAase (13.7 kDa, 300 ng/ cm^2 on silica), bovine serum albumen (66.5 kDa, 150 ng/ cm^2 on silica),⁴⁷ and human serum albumin (66.5 kDa, 230 ng/ cm^2 on oxidized gold).⁴⁸ An estimate of the mass of a closest packed DJ-1 monolayer can also be derived from the dimensions of this protein previously reported using single crystal X-ray diffraction.⁴⁹ The refined crystallographic data include 323 structural water molecules per DJ-1 protein, which should be considered a lower bound to the actual water content of this system. The mass of this monolayer is predicted to be 146 ng/ cm^2 .

The low end (146 ng/ cm^2) and high end of these estimates (300 ng/ cm^2) are indicated by dashed lines in Figure 12c. On the basis of these numbers, the ~ 4 $\mu\text{g}/\text{cm}^2$ plateau measured for DJ-1 corresponded to 13–27 equiv protein monolayers. These data are consistent with permeation of DJ-1 into the virus-PEDOT layer, and perhaps the PEDOT–PSS layer as well, on the one-minute time scale as required by the mechanism depicted in Figure 11.

Finally, it is useful to confirm that the diffusion coefficient, $D_{\text{DJ-1}}$, required for DJ-1 permeation by diffusion in one minute is physically realistic. $D_{\text{DJ-1}}$ can be estimated using the equation:⁵⁰ $D_{\text{DJ-1}} = T^2/2t$ where T is the total thickness of the two-layer channel (≈ 150 – 170 nm) and t is the time required for diffusive permeation of DJ-1 (≈ 60 s), which we estimate as the time required for signal generation. The range of calculated $D_{\text{DJ-1}}$ values is $(1.9$ – $2.4) \times 10^{-12}$ cm^2/s , which is approximately 5–6 orders of magnitude slower than the diffusion coefficient for proteins of this size ($D \approx 10^{-7}$ – 10^{-6} cm^2/s)⁵¹ in aqueous electrolyte solutions, qualitatively as expected. Potentially, the estimates of T , derived from SEM

data (Figure 3), could be too low because these values pertain to dried films. However, doubling the estimate of T (≈ 300 – 340 nm) produces $D_{\text{DJ-1}}$ values of $(7.5$ – $9.6) \times 10^{-12}$ cm^2/s within an order of magnitude of the dried values.

To test the signal transduction mechanism for VBRs proposed here, simultaneous measurements of the electrical impedance and direct QCM measurements of the mass loading of protein are required for comparison with the predictions of eq 4. We expect to carry out these measurements soon.

SUMMARY

The VBR is a biosensor that exploits direct electrical communication with virus particles to measure the concentration of protein biomarkers for cancer and disease. These virus particles, which are engineered to recognize and bind a target protein of interest, are entrained within an electrodeposited virus-PEDOT film. The electrical impedance of this film directly produces the signal required for protein quantitation, eliminating the need for any additional amplification. In addition, no reagents such as redox couples are required for these measurements.

The conductive polymer bilayer comprising the VBR channel can be engineered to adjust its sensitivity. In particular, a reduction in the thickness of the PEDOT–PSS bottom layer from 70 to 48 nm significantly increases the resistance of this layer, from ~ 80 Ω to ~ 300 Ω , increasing the VBR signal for HSA (66.5 kDa) by a factor of 3–10-times. Applying this strategy to the detection of a smaller protein, DJ-1 (20.7 kDa) enables the measurement of this bladder cancer marker at concentrations down to 10 pM using a measurement time of 1.0 min in a dip-and-read modality. The extreme simplicity of the VBR allows for its fabrication in three steps, contributing to excellent sensor-to-sensor reproducibility characterized by CoVs below 7% down to 30 pM for DJ-1, across the entire DJ-1 binding curve spanning four orders of magnitude in concentration.

Direct QCM measurement of the mass of the bioaffinity bilayer demonstrates that the equivalent of multiple (>10) monolayers of DJ-1 protein is able to diffuse into this layer from solution within one minute, a process that coincides temporally with the generation of the VBR impedance signal. The resulting QCM binding curve for DJ-1 resembles the binding curve measured by the VBR for this protein. On the basis of this observation as well as other evidence, a simple model is proposed for signal transduction involving the dilution of the PEDOT conductor by insulating protein molecules resulting in an increased resistance for this layer, a mechanism analogous to that operating in carbon/polymer chemiresistor gas sensors.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.0c00534>.

Materials and fabrication of VBR; process windows compliance assessment of VBRs; influence of C_{NaCl} on VBR response in the absence of protein; VBR circuit elemental values corresponding to the Nyquist plots; protein expression and selection of DJ-1 binders; site-directed mutagenesis of DL1; phage propagation and purification procedures (PDF)

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Notes

The authors declare the following competing financial interest(s): R.M.P. and G.A.W. have a financial interest in a company, PhageTech Inc., that is commercializing the technologies described here. J.S.B., M.T., and S.V.S. are employees of PhageTech Inc., and also have a financial interest in the company. The terms of this arrangement have been reviewed and approved by the University of California, Irvine in accordance with its conflict of interest policies.

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