Video Article

Bacterial Detection & Identification Using Electrochemical Sensors

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Abstract

Electrochemical sensors are widely used for rapid and accurate measurement of blood glucose and can be adapted for detection of a wide variety of analytes. Electrochemical sensors operate by transducing a biological recognition event into a useful electrical signal. Signal transduction occurs by coupling the activity of a redox enzyme to an amperometric electrode. Sensor specificity is either an inherent characteristic of the enzyme, glucose oxidase in the case of a glucose sensor, or a product of linkage between the enzyme and an antibody or probe.

Here, we describe an electrochemical sensor assay method to directly detect and identify bacteria. In every case, the probes described here are DNA oligonucleotides. This method is based on sandwich hybridization of capture and detector probes with target ribosomal RNA (rRNA). The capture probe is anchored to the sensor surface, while the detector probe is linked to horseradish peroxidase (HRP). When a substrate such as 3,3',5,5'-tetramethylbenzidine (TMB) is added to an electrode with capture-target-detector complexes bound to its surface, the substrate is oxidized by HRP and reduced by the working electrode. This redox cycle results in shuttling of electrons by the substrate from the electrode to HRP, producing current flow in the electrode.

Video Link

The video component of this article can be found at https://www.jove.com/video/4282/

Introduction

Using rRNA as a target molecule for bacterial detection and identification has a number of advantages. The abundance of rRNA in bacterial cells provides for a sensitivity limit as low as 250 bacteria per milliliter without the need for target amplification [1]. Bacterial rRNA contains unique species-specific sequences that are accessible to hybridization with DNA probes. Consequently, an array of electrochemical sensors can be used to identify unknown bacteria, where each sensor is functionalized with a different species-specific capture probe. Positive control sensors should be included for a synthetic oligonucleotide target that "bridges" the capture and detector probes to create an internal calibration signal.

Electrochemical sensors have a wide range of basic and translational research applications. For example, the assay described here has been used to precisely measure the effect of *E. coli* growth phase on rRNA and pre-rRNA copy numbers, which is of great interest to researchers interested in bacterial physiology [2]. The sensitivity of the electrochemical sensor assay is determined by the signal to noise ratio. A variety of signal amplification and noise reduction methods have been explored. We find that improving the chemistry of the sensor surface is key to reducing nonspecific binding of detector probe and/or HRP enzyme. In particular, a mixed monolayer of alkanedithiols and mercaptohexanol has been found to reduce background by covering the electrode surface more completely while retaining accessibility of the capture probe for target hybridization [3]. These surface chemistry treatments are particularly important for assays involving complex biological samples.

Protocol

1. Functionalization of Electrochemical Sensors

1. Prepare the thiolated capture probe at a concentration of 0.05 µM in 300 µM 1,6-hexanedithiol (HDT), 10 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 1 mM EDTA and incubate in the dark at room temperature for 10 min. Incubation of the thiolated capture probe with HDT ensures that the thiol group on the capture probe is reduced, resulting in more consistent results.

2. Apply a stream of nitrogen to bare gold 16 sensor array chip(s) for 5 sec to remove moisture and/or particulates.
3. Apply 6 µl of the HDT-thiolated capture probe mix to the working electrode of all 16 sensors of the sensor array and store the sensor chip(s) in a covered Petri dish at 4 °C overnight. Thiolated capture probes bind directly to the bare gold electrode and the HDT acts to prevent overpacking of the capture probes and keep them in an extended conformation that promotes hybridization with the target.
4. The following day, wash the sensor chip with deionized H_2O for 2-3 sec and dry under a stream of nitrogen for 5 sec.
5. Apply 6 µl of 10 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 1 mM EDTA, 1 mM 6-mercapto-1-hexanol (MCH) to the working electrode of all 16 sensors and incubate for 50 min. This and all subsequent sensor chip incubations are performed in a covered Petri dish at room temperature. MCH acts as a blocking agent, filling in any gaps where the thiolated capture probe or HDT is not present on the electrode surface.

2. Sample Preparation

1. Transfer 1 ml of bacterial culture in the log phase of growth (OD_{600} ≈ 0.1) to a microcentrifuge tube and centrifuge at 16,000 x g for 5 min. Remove the culture supernatant. The bacterial pellet can be processed immediately or stored at -80 °C for later use.
2. Thoroughly resuspend the bacterial pellet in 10 µl of 1 M NaOH by applying the pipette tip to the bottom of the microcentrifuge tube and pipetting up and down several times. Incubate the suspension at room temperature for 5 min.
3. Neutralize the bacterial lysate by adding 50 µl of 1 M Phosphate Buffer, pH 7.2, containing 2.5% bovine serum albumin (BSA) and 0.25 mM of a fluorescein-modified detector probe. Incubate the neutralized lysate for 10 min at room temperature. Fluorescein-modified detector probes hybridize with bacterial rRNA target molecules.

3. Electrochemical Sensor Assay

1. Wash the MCH from the sensor chip with deionized H_2O for 2-3 sec and dry under a stream of nitrogen for 5 sec.
2. Apply 4 µl of neutralized bacterial lysate to the working electrode of each of 14 sensors and incubate for 15 min. Target-detector probe complexes hybridize to immobilized thiolated capture probes.
3. Apply 4 µl of 1 nM bridging oligonucleotide in 1 M Phosphate Buffer, pH 7.2, containing 2.5% BSA and 0.25 µM fluorescein-modified detector probe to 2 positive control sensors (used for signal normalization) and incubate for 15 min.
4. Wash the sensor chip with deionized H_2O for 2-3 sec and dry under a stream of nitrogen for 5 sec.
5. Apply 4 µl of 0.5 U/ml anti-Fluorescein-HRP in 1 M Phosphate Buffer, pH 7.2, containing 0.5% casein to the working electrode of all 16 sensors and incubate for 15 min. The anti-Fluorescein-HRP binds to the immobilized fluorescein-modified detector probes.
6. Wash the sensor chip with deionized H_2O for 2-3 sec and dry under a stream of nitrogen for 5 sec.
7. Apply a film well sticker to the surface of the sensor chip and load into the sensor chip mount. Pipette 50 µl of TMB substrate onto all 16 sensors and close the sensor chip mount.
8. Obtain amperometry and cyclic voltammetry measurements for all 16 sensors using the Helios Chip Reader. Amperometric current is proportional to the rate of TMB reduction on the sensor surface (see **Figure 1**).

Representative Results

We describe an electrochemical assay that is structured similarly to a sandwich ELISA. As shown in **Figure 1**, target ribosomal RNA (rRNA) hybridization with capture and detector probes is developed by a redox reaction catalyzed by HRP conjugated to anti-fluorescein antibody fragments that bind to the 3' fluorescein linkage on the detector probe. An important component of assay sensitivity is the surface chemistry of the gold electrode. We have found that a ternary monolayer consisting of thiolated capture probes, mercaptohexanol, and hexanedithiol significantly improves the signal-to-noise ratio by reducing nonspecific background binding of the reporter protein [3]. The assays described here employ a GeneFluidics 16 sensor array chip shown in **Figure 2**. Each of the sensors in the array contain three electrodes, working, reference, and auxiliary, which have contact points at the edge of the chip [4]. The chip reader has pogo pins that connect with each of the contact points. The chip reader serves as an interface for the sensor array with a potentiometer controlled by a computer algorithm.

Examples of electrochemical sensor current flow are shown in **Figure 3**. Current flow in the sensors is artificially high during the first few seconds after amperometric measurements begin because of the accumulation of oxidized substrate during the time from application of TMB to the sensor surface to initiating the reader algorithm. Current flow quickly reaches a steady state and accurate sensor readings can reliably be obtained within sixty seconds. Sensor assays are typically performed in duplicate as an internal control for sensor variability. We have found that sensor-to-sensor variability is relatively constant, so it is possible to perform 16 different sensor assays in parallel on the GeneFluidics 16-sensor array chip. Positive and negative controls are essential. As a positive control, we include a synthetic "bridging" DNA oligonucleotide that hybridizes to both the capture and detector probes and serves as a synthetic target of known concentration. In this way, results obtained using the bridging oligonucleotide functions as an internal calibration control to minimize chip-to-chip variation.

When testing capture and detector probes, the limit of detection should be determined by serial dilution of a sample of known concentration. As shown in **Figure 4**, there is a linear log-log correlation between sample concentration and signal output over the dynamic range of the assay. The limit of detection is defined as the concentration of target required to generate a signal that is 3.29 standard deviations greater than the mean of background signals [5]. For identification of bacteria in an unknown sample, a panel of relevant capture and detector probe pairs are selected. The choice of probes is determined by the types of bacteria suspected in a particular type of sample. Of course, unusual bacteria can occasionally be found in any sample, so we recommend that a eubacterial probe pair be included that has the capacity to hybridize to conserved rRNA regions of any bacteria. **Figure 5** shows representative results for samples containing *E. coli* and *K. pneumoniae*, which are frequently found in the urine of patients with urinary tract infection. Signals for most of the sensors will be low and similar, and these negative results can be pooled as the background signal.

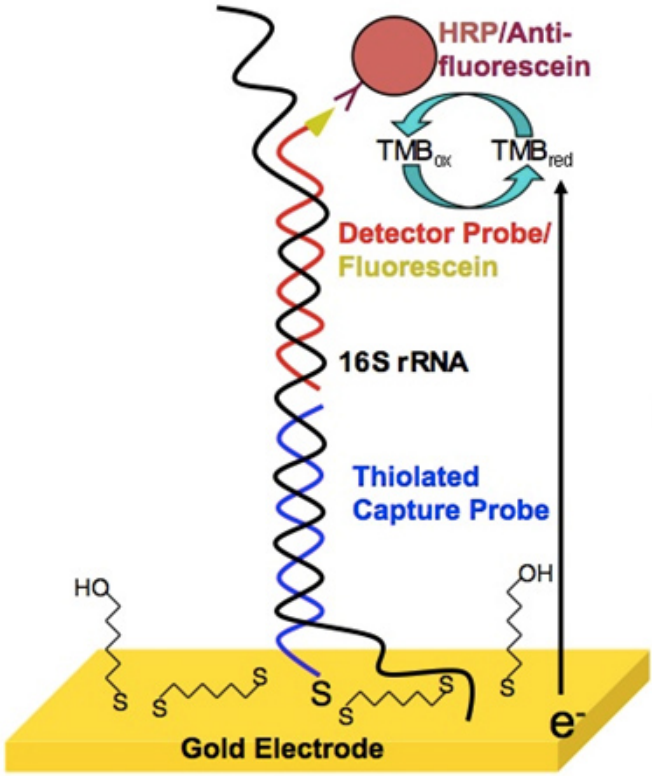

Figure 1. Schematic diagram of the electrochemical sensor assay. The gold electrode is coated with mercaptohexanol and hexanedithiol and functionalized by addition of thiolated DNA oligonucleotide capture probes (blue). Sandwich hybridization of the target 16S rRNA with the DNA oligonucleotide capture probe and fluorescein-labelled DNA oligonucleotide detector probe (red), results in a complex that is detected by addition of anti-fluorescein - horseradish peroxidase (HRP) conjugate and TMB substrate. Amperometric current is generated by the redox cycle that results from oxidation of TMB by HRP and reduction of TMB by electrons from the electrode.

Figure 2. Bacterial identification sensor array and chip mount. Each sensor in the array consists of three electrodes: a central working electrode, a circumferential reference electrode and an auxiliary electrode for signal stabilization. The working electrodes of each sensor in the array are functionalized with a panel of capture probes specific for bacterial species of interest (see **Table 3**) as well as an EU (eubacterial) capture probe that hybridizes to all bacterial 16S rRNA molecules and a BrOI "bridging" oligonucleotide that functions as positive control for signal calibration. Signal is measured by placing the array in a chip reader with pogo pins that interface with the sensor electrode contact points.

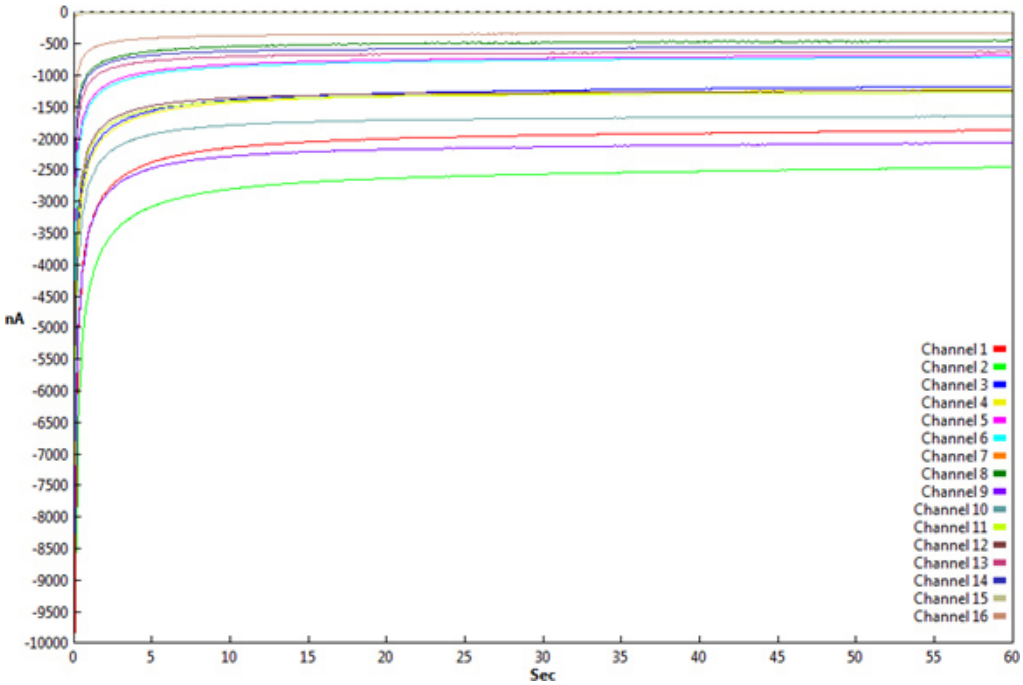

Figure 3. Amperometric current output during measurement. Amperometric current is measured in parallel for all 16 sensors in the array. Current flow is initially extremely negative due to accumulation of oxidized TMB substrate prior to connecting the array to the chip reader and starting measurement. Thereafter, current quickly stabilizes and sensor readings are recorded at 60 sec. Electrodes with greater negative current have the highest signals.

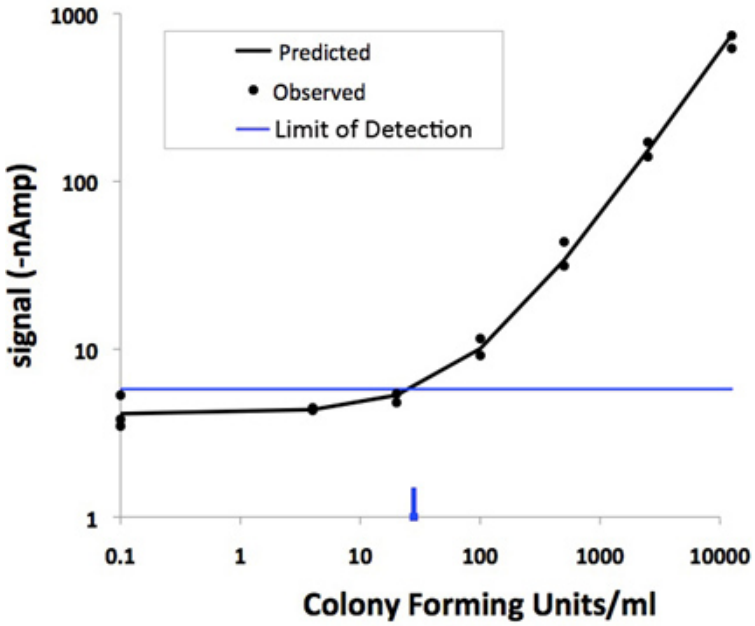

Figure 4. Serial tenfold dilution of *E. coli* to determine the limit of detection. Sensors were tested in duplicate with serial tenfold dilutions of a known concentration of *E. coli* lysate. The observed signal readings, plotted as black circles, were used to predict an idealized signal curve. The pooled standard deviation for all replicates and the background signal at the lowest target concentration were used to calculate the limit of detection (blue line), defined as 3.29 standard deviations over background.

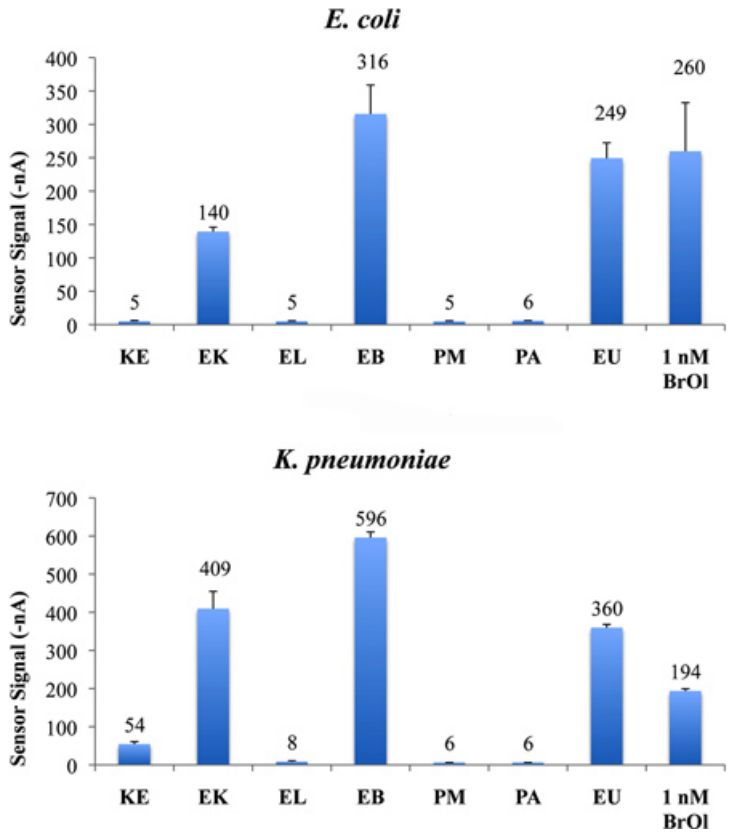

Figure 5. Representative electrochemical sensor assay results for *Escherichia coli and Klebsiella pneumoniae*. *E. coli* and *K. pneumoniae* were tested for reactivity with probe pairs described in **Tables 2** and **3**. Reactivity with the KE probe pair distinguishes *K. pneumoniae* from *E. coli.* The bridging oligonucleotide (BrOl) serves as positive control and internal calibration standard.

Probe Name	Ribosomal Subunit and Location*	Sequence
KE Cap 10m	16S 74	5'-S-AGCTCTCTGT
KE Det 11m	16S 63	5'-GCTACCGYTCG-F
EK Cap 15m	23S 1492	5'-AGCCTTGATTTTCCG-S
EK Det 15m	23S 1507	5'-F-TCGTCATCACACCTC
EL Cap 17m	23S 1507	5'-AGCGTTAAAAGGTACCG-S
EL Cap 18m	23S 1507	5'-AGCGTTAADAAGGTACCG-S
EL Det 15m	23S 1492	5'-F-TCGTCATCACACCTC
EB Cap 23m	16S 1275	5'-S-ACTTTATGAGGTCCGCTTGCTCT
EB Det 23m	16S 1252	5'-CGCGAGGTCGCCTTCCTTTGTAT-F
PM Cap 15m	16S 183	5'-S-TTTGGTCCGTAGACA
PM Det 17m	16S 166	5'-TTATGCGGTATTAGCCA-F
PA Cap 11m	16S 453	5'-S-ACTTACTGCCC
PA Det 14m	16S 439	5'-TTCCTCCCAACTTA-F
EU GN Cap 19m	16S 1502	5'-S-GTTCCCCTACGGTTACCTT
EU Det 18m	16S 1484	5'-GTTACGACTTCACCCCAG-F
EU BrOl 37m		5'-CTGGGGTGAAGTCGTAACAAGGTAACCGTAGGGGAAC

Table 2. DNA Oligonucleotide Probe Pair Sequences. Capture (Cap) probes are synthesized with thiol linkages (S). Detector (Det) probes are synthesized with fluorescein (F) modifications. Note that probe pairs involve either a 5'-thiolated capture probe and a 3'-fluorescein-modified detector probe or a 3'-thiolated capture probe and a 5'-fluorescein-modified detector probe. The two EL capture probes are mixed prior to use. To simplify the assay, detector probes can be applied to each sensor as a mixture without significant cross-reactivity. KE - *Klebsiella* and

Enterobacter, EK - *E.coli* and *K. pneumoniae*, EL - *E. cloacae*, EB - *Enterobacteriaceae* family, PM - *P. mirabilis*, PA - *P. aeruginosa*, EU - Eubacterial, BrOl - Bridging Oligonucleotide. * - Location number refers to the distance from the start of the 16S or 23S gene.

Bacterial Species	Reactive Probe Pairs
Escherichia coli	EK + EB
Klebsiella pneumoniae	KE + EK + EB
Proteus mirabilis	PM + EB
Pseudomonas aeruginosa	PA
Enterobacter species	KE + EB or EL + EB or KE + EL + EB

Table 3. Recognition of Bacterial Species by Probe Pairs.

Discussion

The electrochemical sensor assay described here enables rapid detection of nucleic acid targets. Sensitivity and specificity depend in part on the free energy of target-probe hybridization, which in turn depends on the length and GC content of the capture and detector probes. We typically perform the hybridization steps at ambient temperature (~20 °C) [5, 6]. However, the hybridization steps (3.2 and 3.3) can also be performed at higher temperatures in a hybridization oven if the chip is placed in a covered chamber containing moistened filter paper to prevent evaporation [7, 8]. Optimal signal is obtained with capture-detector probe pairs that hybridize to adjacent sites on the nucleic acid target without a gap between the hybridization sites [8]. This signal enhancement is thought to work by base-pair stacking and by increasing hybridization accessibility of adjacent regions, known as a "helper probe" effect. Capture-detector probe hybridization to adjacent sites is also important because alkaline lysis of the bacteria results in fragmentation of the rRNA target [5]. This method can be applied to detection of either RNA or DNA targets, with a wide variety of clinical and research applications. For example it should be possible to use this method to study cellular functions that are regulated by small RNAs or to detect antibiotic resistance gene targets either as either DNA or mRNA. Pre-amplication may be necessary to detect targets present at low copy numbers. In addition, RNAse inhibitors should be added during sample preparation to prevent degradation of potentially unstable RNA targets.

This method has several advantages over other methods of detecting and identifying bacteria. Standard clinical microbiology methods require bacterial growth on agar medium for quantitation and application of identification methods. Growth of bacteria to visible colonies typically involves overnight incubation, significantly delaying the time from specimen collection to reporting of results. There is great interest in development of more rapid, molecular methods of pathogen detection, including electrochemical biosensors [9]. However, little attention has been given to the performance of DNA biosensors in raw biological samples. The electrochemical sensor assay described here can be performed directly on serum and urine samples without a significant loss of sensitivity [7]. The use of thiolated capture probes and the mixed monolayer described here renders the sensor surface resistant to non-specific protein absorption, retaining sensitivity in raw biological specimens including serum or urine [10]. As few as 1 bacterial cell per sensor (250 bacteria per milliliter) are detectable, which is comparable to the sensitivity of PCR amplification techniques [1]. Several PCR based bacterial identification methods have been described [11, 12]. Although PCR can potentially detect fewer target molecules than the electrochemical sensor assay described here, PCR-based methods have achieved limited adoption because of the high equipment acquisition and/or reagent costs, relative to standard clinical microbiology methods. In contrast, electrochemical sensors are relatively inexpensive to produce. Even though the electrodes are fabricated by gold sputtering, the amount of gold per chip is low because the electrode thickness is only 1,000 Ângströms.

Disclosures

All authors are inventors on patents relevant to the methods described. One of these patents has been licensed to Qvella Corporation. B.M.C. and D.A.H. have equity interest in Qvella Corporation. V.G. is the President of GeneFluidics, which is the manufacturer of the electrochemical sensor array chip, chip mount, and chip reader described in this paper.

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