Technical Advance

Development of an Advanced Electrochemical DNA Biosensor for Bacterial Pathogen Detection

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Electrochemical sensors have the capacity for rapid and accurate detection of a wide variety of target molecules in biological fluids. We have developed an electrochemical sensor assay involving hybridization of bacterial 16S rRNA to fluorescein-modified detector probes and to biotin-modified capture probes anchored to the sensor surface. Signal is generated by an oxidation-reduction current produced by the action of horseradish peroxidase conjugated to an anti-fluorescein monoclonal Fab. A previous study found that this electrochemical sensor strategy could identify uropathogens in clinical urine specimens. To improve assay sensitivity, we examined the key steps that affect the current amplitude of the electrochemical signal. Efficient lysis and release of 16S rRNA from both gram-negative and -positive bacteria was achieved with an initial treatment with Triton X-100 and lysozyme followed by alkaline lysis, resulting in a 12-fold increase in electrochemical signal compared with alkaline lysis alone. The distance in nucleotides between the target hybridization sites of the detector and capture probes and the location of fluorescein modification on the detector probe contributed to a 23-fold change in signal intensity. These results demonstrate the importance of target-probe and probe-probe interactions in the detection of bacterial 16S rRNA using an electrochemical DNA sensor approach. (*J Mol Diagn* 2007, 9:158–168; DOI: 10.2353/jmoldx.2007.060052)

cleic acids.^{1–5} The ability of electrochemical sensors to directly identify nucleic acids in complex mixtures is a significant advantage over approaches such as polymerase chain reaction (PCR) that require target purification and amplification. Application of DNA sensor technology to infectious diseases has the potential for recognition of pathogen-specific signature sequences in biological fluids. The most frequent body fluid submitted to clinical microbiology laboratories is urine. Culture-based methods for isolation and identification of uropathogens are time consuming and labor intensive, contributing significantly to the estimated \$3.5 billion annual cost of treating urinary tract infections in the United States.^{6,7} A rapid, automated point-of-care system for identification of bacterial pathogens would have a significant impact on the clinical management of urinary tract infection (UTI) and infectious diseases in general.

A general approach for species-specific identification of bacterial pathogens using an electrochemical sensor involves hybridization of single-stranded oligonucleotide capture and detector probes to target 16S rRNA.^{2,8} The capture probe anchors the target to the sensor, whereas the detector probe signals the presence of the target through a reporter molecule (Figure 1). Binding of the capture and detector probes to the nucleic acid target creates a three-component “sandwich” complex on the sensor surface.^{9–12} The fluorescein-modified detector probe enables binding of an anti-fluorescein-conjugated horseradish peroxidase reporter enzyme to the target-probe complex.⁸ The addition of a redox substrate and application of a fixed potential between the working and reference sensor electrodes creates a horseradish peroxidase-mediated redox cycle that is detected by the electrochemical sensor as current.^{13,14} In this way, the amplitude of the electroreduction current reflects the con-

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Microfabrication technology has enabled the development of electrochemical DNA biosensors with the capacity for sensitive and sequence-specific detection of nu-

Figure 1. Flow chart showing the steps involved in the process of bacterial detection using the electrochemical sensor. Step 1: Bacterial lysis to release the 16S rRNA target. Step 2: Primary hybridization of the 16S rRNA target with the fluorescein-modified detector probe. Step 3: Secondary hybridization of the target-detector probe hybrid to the capture probe on the sensor surface. Step 4: Redox reaction generated by addition of anti-fluorescein horseradish peroxidase (α F-HRP), TMB substrate, and H_2O_2 . The amount of time required for each step is shown. Washing occurs only before and after the addition of α F-HRP. The entire assay was performed within 50 minutes.

centration of the target-probe complexes on the sensor surface.

Initial proof-of-concept research has indicated that this electrochemical sensor approach has the potential for species-specific detection of uropathogens in clinical urine specimens.^{15,16} However, the sensitivity of the detection system seems to be limited by inefficiencies in bacterial lysis and probe-target hybridization. In this study, we have conducted a detailed examination of the determinants of electrochemical signal intensity, namely 1) bacterial lysis and release of the 16S rRNA target molecules, 2) the length of the capture-detector probe complex, 3) the distance between the capture and detector probe hybridization regions on the 16S rRNA target, 4) the location of fluorescein on the detector probe, and 5) probe-probe and probe-target interactions during hybridization with mixtures of detector probes. Major improvements in signal intensity were achieved, contributing significantly toward our goal of developing a microfluidics-based "lab-on-a-chip" electrochemical sensor assay for bacterial pathogen detection.

Materials and Methods

Bacterial Strains and Cultivation

The following American Type Culture Collection (ATCC) strains were obtained from the University of California-

Los Angeles (UCLA) Clinical Microbiology Laboratory: *Escherichia coli* strain 35218, *Klebsiella pneumoniae* strain 13883, *Klebsiella oxytoca* strain 49131, *Enterobacter aerogenes* strain 13048, *Enterobacter cloacae* strain 13047, *Proteus mirabilis* strain 12453, *Pseudomonas aeruginosa* strain 10145, *Citrobacter freundii* strain 8090, and *Enterococcus faecalis* strain 49532. Additional strains of uropathogenic bacteria were obtained from the UCLA Uropathogen Specimen Bank: *E. coli* strain Ec103, *K. pneumoniae* strain Kp295, *P. mirabilis* strain Pm278, *P. aeruginosa* strain Pa3, and *E. faecalis* strain Eo111. Isolation of uropathogens from clinical urine specimens was approved by the UCLA and VA Greater Los Angeles Healthcare System Institutional Review Boards. The identity of all clinical strains was determined by standard biochemical assays and verified by 16S rRNA gene sequencing. The 16S rRNA genes were PCR amplified with universal primers 8UA and 1485B.¹⁷ The amplified product was purified by using the QIAquick PCR purification kit (Qiagen, Inc., Chatsworth, CA) and directly sequenced using primer pairs 8UA/907B and 774A/1485B as described previously.¹⁸ DNA sequencing was performed at the W.M. Keck Foundation Biotechnology Resource Laboratory (New Haven, CT). Isolates were inoculated into *Brucella* broth with 15% glycerol (BBL, Annapolis, MD) and were stored at -70°C . All experiments reported here involved bacteria grown overnight in Luria broth, inoculated into Luria broth, and grown to logarithmic phase as measured by OD_{600} . Concentration of the logarithmic phase specimens was determined by serial plating, typically yielding 10^7 to 10^8 bacteria/ml.

Oligonucleotide Probe Design

Oligonucleotide probes were synthesized by MWG Biotech (High Point, NC). Capture probes were synthesized with a 5' biotin modification. Detector probes were synthesized with 5'- and/or 3'-fluorescein modifications. Oligonucleotide probe pairs were designed to hybridize with species-specific regions of the 16S rRNA molecules of *E. coli*, *E. faecalis*, *P. mirabilis*, and *P. aeruginosa*. Oligonucleotides were also designed as capture and detector probes for the *Klebsiella-Enterobacter* group, the family *Enterobacteriaceae*, and as universal bacterial probes. Probe pairs were studied with and without a gap between the hybridization regions on the 16S rRNA target. The sequences of all oligonucleotide probes used in this study are shown in Table 1.

Sensor Characterization and Surface Functional Layer Preparation

Microfabricated electrochemical sensor arrays with an alkanethiolate self-assembled monolayer were obtained from GeneFluidics (Monterey Park, CA). Self-assembled monolayer integrity was confirmed by cyclic voltammetry¹⁹ using a 16-channel potentiostat (GeneFluidics). After cyclic voltammetry characterization, sensor arrays were washed and dried. Washing steps were performed by applying a stream of deionized H_2O to the sensor

Table 1. Sequences of Oligonucleotide Probes Used in This Work

Probe designation*	Sequence (5'-3')
Capture probes	
EB1172C (35-mer)	5'-CGGACTACGACRYACTTTATGAGGTCCGCTTGCTC-3'
EC434C (35-mer)	5'-GTCAATGAGCAAAGGTATTAACCTTTACTCCCTTCC-3'
EC430C (35-mer)	5'-GAGCAAAGGTATTAACCTTTACTCCCTTCCCTCCCG-3'
EF207C (35-mer)	5'-TTGGTGAGCCGTTACCTCACCAGCTAGCTAATGCA-3'
EF165C (35-mer)	5'-GTCCATCCATCAGCGACACCCGAAAGCGCCTTTCA-3'
KE434C (35-mer)	5'-GTCAATCGMCRAGGTATTAACCTYAHCGCCTTCC-3'
PA111C (35-mer)	5'-CCCACTTTCTCCCTCAGGACGTATGCGGTATTAGC-3'
PA972C (35-mer)	5'-TGAGTTCCCGAAGGCACCAATCCATCTCTGGAAG-3'
PM188C (35-mer)	5'-GGGTTTCATCCGATAGTGCAAGGTCCGAAGAGCCCC-3'
UNI782C (27-mer)	5'-CATCGTTTACGCGGTGGACTACCAGGG-3'
Detector probes	
EB1137D (35-mer)	5'-GAGGTCGCTTCTCTTTGTATRYGCCATTGTAGCAC-3'
EB1141D (35-mer)	5'-TCGCGAGGTCGCTTCTCTTTGTATRYGCCATTGTA-3'
EC393D (35-mer)	5'-CTGAAAGTACTTTACAACCCGAAGGCCTTCTTCAT-3'
EC394D (35-mer)	5'-GCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCA-3'
EC395D (35-mer)	5'-CGTGAAAGTACTTTACAACCCGAAGGCCTTCTTC-3'
EC396D (35-mer)	5'-CCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTT-3'
EC397D (35-mer)	5'-CCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCT-3'
EC398D (35-mer)	5'-CCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCT-3'
EC399D (35-mer)	5'-TCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTT-3'
EF165D (35-mer)	5'-GTCCATCCATCAGCGACACCCGAAAGCGCCTTTCA-3'
EF171D (35-mer)	5'-CCGCGGGTCCATCCATCAGCGACACCCGAAAGCGC-3'
EF207D (35-mer)	5'-TTGGTGAGCCGTTACCTCACCAGCTAGCTAATGCA-3'
KE393D (35-mer)	5'-CTGAAAGTGCTTTACAACCCGAAGGCCTTCTTCAT-3'
KE399D (35-mer)	5'-TCCCCGCTGAAAGTGCTTTACAACCCGAAGGCCTT-3'
PA68D (35-mer)	5'-TTCGGGACGTTATCCCCCACTACCAGGCAGATTCC-3'
PA74D (35-mer)	5'-GCCCCGTTTCCGGACGTTATCCCCCACTACCAGGCA-3'
PA932D (35-mer)	5'-CAGCATGTCAAGGCCAGGTAAGGTCTTCGCGTTG-3'
PM147D (35-mer)	5'-GGTCCGTAGACATTATGCGGTATTAGCCACCGTTT-3'
PM153D (35-mer)	5'-TGCTTTGGTCCGTAGACATTATGCGGTATTAGCCA-3'
UNI751D (31-mer)	5'-TATCTAATCCTGTTTGCTCCCCACGCTTTTCG-3'

*Capture probes were 5'-modified with biotin. Detector probes were 5'- and/or 3'-modified with fluorescein. Probe sequence numbering based on *E. coli* 16S rDNA. EB, *Enterobacteriaceae* family; EC, *Escherichia coli*; EF, *Enterococcus* species; KE, *Klebsiella-Enterobacter* group; PA, *Pseudomonas aeruginosa*; PM, *Proteus mirabilis*; UNI, universal bacterial.

surface for approximately 2 to 3 seconds followed by 5 seconds of drying under a stream of nitrogen. To functionalize the sensor surface, 4 μ l of 0.5 mg/ml streptavidin (Calbiochem, San Diego, CA) in H₂O was added to the alkanethiol-activated sensors, incubated for 10 minutes at room temperature, and washed off. Biotinylated capture probes (4 μ l, 1 μ mol/L in 1 mol/L phosphate buffer, pH 7.4) were added to the streptavidin-coated sensors. Phosphate buffer (1 mol/L), pH 7.4, was prepared by mixing 1 mol/L NaH₂PO₄ and 1 mol/L K₂HPO₄ in a 19:81 (v/v) ratio, respectively, and adjusting the pH to 7.4. After 30 minutes of incubation at room temperature, the sensor array was washed and dried, completing the surface preparation.

Amperometric Detection of Bacterial 16S rRNA

Logarithmic phase bacterial cells were concentrated by centrifugation at 10,000 rpm for 5 minutes. Lysis of bacterial cells was performed by the addition of 10 μ l of one or more of the following: 1 mol/L NaOH, 0.1% Triton X-100 in 20 mmol/L Tris-HCl, pH 8.0, 2 mmol/L ethylenediamine tetraacetic acid, and 1 mg/ml lysozyme (Sigma, St. Louis, MO). After incubation at room temperature, 50 μ l of the detector probe (0.25 μ mol/L) in 2.5% bovine serum albumin (Sigma) and 1 mol/L phosphate buffer, pH 7.4, was added to the bacterial lysate. The detector probe/bacte-

rial lysate mixture was incubated for 10 minutes at 65°C to allow hybridization of the detector probe to target rRNA. Four microliters of the bacterial lysate/detector probe mixture was deposited on each of the working electrodes in the sensor array. The sensor array was incubated for 15 minutes at 65°C in a humidified chamber. After washing and drying, 4 μ l of 0.5 U/ml anti-fluorescein horseradish peroxidase (HRP) Fab conjugate (diluted in 0.5% casein in 1 mol/L phosphate buffer, pH 7.4; Roche Diagnostics, Mannheim, Germany) were deposited on each of the working electrodes for 10 to 15 minutes. After washing and drying, a prefabricated plastic well manifold (GeneFluidics) was bonded to the sensor array. HRP substrate solution (50 μ l of K-Blue Aqueous TMB; Neogen, Lexington, KY) was placed on each of the sensors in the array to cover all three of the electrodes. Measurements were immediately and simultaneously taken for all 16 sensors. The entire assay protocol was completed within 45 minutes from the initiation of bacterial lysis. Amperometric current versus time was measured using a multichannel potentiostat (GeneFluidics). The voltage was fixed at -200 mV (versus reference), and the electroreduction current was measured at 60 seconds after the HRP redox reaction reached steady state. Negative controls were included in each experiment in which 1 mol/L phosphate buffer, pH 7.4, was

Figure 2. Effect of lysis conditions on electrochemical signal intensity. *Enterococcus* cells (10^5) were exposed to NaOH, Triton X-100 (Tx), and/or lysozyme (Lzm) at room temperature followed by direct electrochemical detection of released 16S rRNA using *Enterococcus*-specific capture (EF207C) and detector (EF165D) probes. The lysis conditions were as follows: 1) NaOH for 10 minutes; 2) Triton X-100 for 5 minutes, followed by NaOH for 5 minutes; 3) Triton X-100 and lysozyme for 10 minutes; 4) NaOH for 5 minutes, followed by Triton X-100 and lysozyme for 5 minutes; and 5) Triton X-100 and lysozyme for 5 minutes, followed by NaOH for 5 minutes. Mean and SD of experiments performed in duplicate are shown. Background signal was determined in negative control experiments performed with capture and detector probes but without bacterial lysate. Conditions 3, 4, and 5 produced results that were significantly greater than negative control ($P < 0.01$).

used as the target instead of bacterial lysate. All samples were analyzed in duplicate.

Experiments were performed on ATCC strains to verify probe specificity using a 16-sensor array "UTI chip" in which the UNI782C, EB1176C, EC434C, KE434C, PM187C, PA102C, and EF207C 5'-biotinylated capture probes (defined in Table 1) were tested in duplicate. The two remaining sensors in the array served as negative controls (using capture probe UNI782C in 1 mol/L phosphate buffer, pH 7.4, instead of bacterial lysate). Bacterial lysates were combined with a mixture of the following 3'-fluorescein-labeled detector probes: UNI751D, EB1141D, EC399D, KE399D, PM153D, PA74D, and EF171D (defined in Table 1). The degree of variance in the electrochemical sensor measurements was determined by comparing duplicate measurements for all experiments. The background signal level was determined by averaging the $\log_{10}$ results of the two negative control sensors. Positive signals were defined as signals greater than 5 SD (in $\log_{10}$ units) over background.

Results

Universal Bacterial Lysis Strategy for Release of 16S rRNA

An effective 16S rRNA detection system for uropathogens requires efficient lysis of gram-negative and gram-positive organisms. Previous work had shown that alkaline lysis efficiently released 16S rRNA from gram-negative but not gram-positive bacteria.¹⁶ For this reason, we examined a variety of methods for rapid

Figure 3. Electrochemical signal intensity as a function of the distance (in nucleotides) between the capture and detector probe hybridization sites on the 16S rRNA target. Current output (in nanoamperes) was measured using capture probe EC434C and various 3'-fluorescein-modified detector probes hybridized to 16S rRNA released from 4.2×10^7 *E. coli*. Mean and SD of experiments performed in duplicate are shown. Background signal was determined in negative control (NC) experiments performed with capture and detector probes but without bacterial lysate. There was a negative correlation ($r = -0.84$) between signal intensity and the number of nucleotides between the capture and detector probe hybridization sites. The signal intensity obtained using capture and detector probes hybridizing to adjacent (0-nucleotide gap) sites produced an electrochemical signal significantly greater than that obtained using detector probes hybridizing ≥ 3 nucleotides away from the capture probe hybridization site ($P < 0.01$).

release of 16S rRNA from the gram-positive uropathogen, *E. faecalis*. Given the thicker cell wall of gram-positive organisms, we considered whether membrane-active detergents and/or peptidoglycan-specific enzymes would be useful components of an effective lysis strategy. As shown in Figure 2, NaOH with or without the detergent Triton X-100 did not lyse *Enterococcus* cells sufficiently for electrochemical detection of 16S rRNA above negative control signal levels. However, the combination of 0.1% Triton X-100 plus 1 mg/ml lysozyme resulted in a threefold increase in current output over negative control. The approach that yielded the highest electrochemical signal intensity was a strategy in which *Enterococcus* cells were initially treated with the combination of Triton X-100 and lysozyme for 5 minutes followed by treatment with NaOH for an additional 5 minutes. This two-step lysis method resulted in a 12-fold increase in electrochemical signal compared with alkaline lysis alone. The sequence in which these treatments were applied was important. Alkaline lysis before application of Triton X-100 and lysozyme was not as effective as treatment with NaOH after the detergent-enzyme combination. Longer periods of lysis did not further enhance signal intensity. Lysis of gram-negative uropathogens (eg, *E. coli*, *P. mirabilis*, *K. pneumoniae*, and *P. aeruginosa*) with Triton X-100 and lysozyme or Triton X-100 and lysozyme followed by NaOH resulted in successful electrochemical detection of 16S rRNA, although the results were not significantly better than lysis with NaOH alone (data not shown). Therefore, this two-step process can be considered a universal lysis strategy for

Table 2. Effect of a Gap between the Capture and Detector Probe Hybridization Sites

Target*	Probe pair (6-nucleotide gap)	nA $\pm$ SD	Probe pair (0-nucleotide gap)	nA $\pm$ SD	Fold change (0:6 nucleotide)
Ec	EC 434C/393D [†]	590 $\pm$ 65	EC 434C/399D [†]	3242 $\pm$ 85	5.49 [‡]
Ef	EF 207C/165D [†]	1202 $\pm$ 85	EF 207C/171D [†]	1777 $\pm$ 57	1.48
Kp	KP 434C/393D [†]	2106 $\pm$ 186	KE 434C/399D [†]	7789 $\pm$ 93	3.70 [‡]
Kp	KP 972C/932D [§]	1438 $\pm$ 151	KE 972C/938D [§]	2975 $\pm$ 610	2.07 [¶]
Pa	PA 102C/68D [†]	2483 $\pm$ 85	PA 102C/74D [†]	5455 $\pm$ 35	2.20
Pm	PM 187C/147D [†]	908 $\pm$ 19	PM 187C/153D [†]	3361 $\pm$ 248	3.70 [‡]

*Ec, *E. coli*; Ef, *E. faecalis*; Kp, *K. pneumoniae*; Pa, *P. aeruginosa*; Pm, *P. mirabilis*.

[†]3'-Fluorescein-modified detector probe.

[‡] $P < 0.001$.

[§]5'-Fluorescein-modified detector probe.

[¶] $P < 0.05$.

^{||} $P < 0.01$.

release of bacterial 16S rRNA and was used for the remainder of the experiments described here involving *E. faecalis*. For the sake of simplicity, subsequent experiments involving gram-negative organisms involved lysis with NaOH alone. Use of various concentrations of the denaturing detergent sodium dodecyl sulfate coupled with nonspecific proteases (eg, proteinase K or Pronase) did not further improve electrochemical signal strength (data not shown).

Effect of Distance between Capture and Detector Probe Hybridization Sites on Signal Intensity and Sensitivity

The effect of distance between the capture and detector probe hybridization sites on the 16S rRNA target was examined. Experiments involving a variety of 16S rRNA targets using capture and detector probes with hybridization

A. *Enterococcus faecalis*

B. *Escherichia coli*

Figure 4. Electrochemical signal intensity as a function of location of probe hybridization and fluorescein modification. **A:** Signal intensity was measured using the *Enterococcus*-specific capture probe EF C207 paired with 3'- or 5'-fluorescein-modified detector probes hybridizing to the enterococcal 16S target at a site adjacent to (EF D171) or six nucleotides removed from (EF D165) the capture probe. **B:** Signal intensity was measured using 3'- or 5'-fluorescein-modified detector probe EC393D paired with the *E. coli*-specific capture probes hybridizing to the *E. coli* 16S target at a site adjacent to (EC430C) or six nucleotides removed from (EC434C) the detector probe. The configurations of the capture and detector probes are shown schematically. Mean and SD of experiments performed in duplicate are shown.

A *Enterococcus faecalis* B *Escherichia coli*

Figure 5. Sensitivity of the electrochemical sensor assay as a function of locations of probe hybridization and fluorescein modification. **A:** Electrochemical sensor results from fivefold serial dilutions of enterococcal cells using capture probe EF207C paired with detector probes EF165D or EF171D modified by fluorescein at the 5' and 3' positions, respectively. **B:** Electrochemical sensor results from fivefold serial dilutions of *E. coli* cells using capture probe EC434C paired with detector probes EC393C or EC399C modified by fluorescein at the 5' and 3' positions, respectively. The dashed horizontal lines indicate current output thresholds for duplicate results significantly greater than negative control ($P < 0.01$). 3'-Fluorescein modification of the detector probe combined with continuity between the detector and capture probe hybridization sites resulted in a 23-fold improvement in sensitivity of electrochemical sensor assay for detection of enterococci (from 232,000 down to 9900 cells) and *E. coli* (from 6500 down to 280 cells).

sites separated by a gap of >300 nucleotides produced no significant electrochemical current output, even though these probes were known to function well as members of juxtaposed probe pairs (data not shown). In contrast, capture and detector probes with hybridization sites separated by relatively short interprobe gaps of up to six nucleotides yielded positive signals. As shown in Figure 3, there was a negative correlation (Pearson product-moment correlation coefficient $r = -0.84$) between signal intensity and the size of the gap between the capture and detector probe hybridization sites, even for very short interprobe gaps. Maximal signal intensity required eliminating the interprobe hybridization site gap between probe pairs specific for the *Klebsiella-Enterobacter* group, *P. aeruginosa*, and *P. mirabilis* (Table 2), which bind to various regions of the 16S rRNA target. These results indicate that the effect of an interprobe gap on signal intensity is independent of the 16S rRNA target hybridization site and species of origin.

Effect of Location of Fluorescein-Modification on the Detector Probe

We considered whether the location of the fluorescein (the binding site for the anti-fluorescein Fab-HRP conjugate) on the detector probe affected signal intensity. To examine this question, we compared the signal intensity produced using 3'- versus 5'-fluorescein-modified detector probes. Use of 3'-fluorescein-modified detector probes resulted in greater signal intensity than 5'-fluorescein-modified detector probes for detection of both *E. faecalis* (Figure 4A) and *E. coli* (Figure 4B) 16S rRNA. As shown in Figure 4, there was

an additive effect of removing the interprobe gap between the capture and detector probe hybridization sites and moving the fluorescein modification from the 5' to the 3' end of the detector probe. Experiments using fivefold dilutions of enterococcal and *E. coli* cells showed that the probe pair with a 0-nucleotide gap and a 3'-fluorescein-modified detector probe had a 23- to 29-fold lower limit of detection sensitivity compared with the probe pair with a 6-nucleotide gap and a 5'-fluorescein-modified detector probe (Figure 5).

As shown in Table 3, we examined whether the effect of the location of fluorescein modification could be generalized for a variety of detector probes and targets. Some of the detector probes (UNI751D, EB1137D, and EC399D) were also modified with fluorescein at both the 5' and 3' positions. Significant increases in electrochemical current output were achieved using fluorescein modification at the 3' position compared with the 5' position for all of the species-specific detector probes. Interestingly, the location of fluorescein modification had no effect on signal intensity in the case of the universal (UNI751D) bacterial detector probe. In addition, fluorescein labeling of the detector probe at both the 3' and 5' positions did not enhance signal strength beyond that achieved with 3' modification alone.

Effects of Detector Probe Mixtures on Signal Intensity

Use of the electrochemical sensor array to identify unknown bacteria in clinical urine specimens would be greatly simplified by including all relevant detector probes in a single

Table 3. Effect of Location of Detector Probe Fluorescein Modification

Target*	Probe pair	5' Fluorescein (nA $\pm$ SD)	3' Fluorescein (nA $\pm$ SD)	Fold change (3':5')
Ef	EF 207C/165D [†]	236 $\pm$ 59	1202 $\pm$ 85	5.10 [‡]
Ef	EF 207C/171D [§]	1093 $\pm$ 32	1777 $\pm$ 57	1.63
Ec	EC 434C/399D [§]	2781 $\pm$ 144	7313 $\pm$ 336	2.63 [¶]
Ec	EC 434C/393D [†]	2993 $\pm$ 674	4288 $\pm$ 603	1.43
Ec	EC 430C/393D [§]	5650 $\pm$ 270	6816 $\pm$ 858	1.21
Ec	EB 1176C/1137D	1122 $\pm$ 35	2528 $\pm$ 144	2.25 [¶]
Ec	UNI 782C/751D [§]	1890 $\pm$ 491	2053 $\pm$ 189	1.09
Pm	PM 188C/147D [†]	420 $\pm$ 58	806 $\pm$ 45	1.92 ^{**}
Pm	UNI 782C/751D [§]	1388 $\pm$ 10	1502 $\pm$ 33	1.08
Pa	PA 111C/68D [†]	668 $\pm$ 73	1295 $\pm$ 238	1.94 ^{**}
Pa	PA 972C/932D [†]	393 $\pm$ 35	1314 $\pm$ 115	3.34 [‡]

*Ec, *Escherichia coli*; Ef, *Enterococcus faecalis*; Pa, *Pseudomonas aeruginosa*; Pm, *Proteus mirabilis*.

[†]Six-nucleotide gap between the capture and detector probe hybridization sites.

[‡] $P < 0.001$.

[§]Zero-nucleotide gap between the capture and detector probe hybridization sites.

[¶] $P < 0.01$.

^{||}Four-nucleotide gap between the capture and detector probe hybridization sites.

^{**} $P < 0.05$.

hybridization step. However, probe-probe and probe-target interactions could potentially reduce the sensitivity and specificity of the sensor when a 16S rRNA target is hybridized with mixtures of detector probes. For this reason, we systematically examined the effect of probe-target hybridization using mixtures of detector probes on signal intensity. A representative experiment is shown in Figure 6 in which enterococcal 16S rRNA was hybridized with a mixture of the EF165D and EC393D detector probes versus hybridization with the EF165D detector probe alone. When the target-detector probe hybrids were applied to sensors functionalized with the EF207C capture probe, there was no significant difference in signal intensity between the results generated with the EF165D + EC393D detector probe mix-

ture and results generated with the EF165D detector probe alone. Hybridization of these same detector and capture probe mixtures with *E. coli* 16S rRNA resulted in no significant signal, indicating that sensor specificity was retained despite hybridization of the *E. coli* 16S rRNA target with the *E. coli*-specific EC393D detector probe. Likewise, no loss of sensitivity or specificity was observed with other two-detector probe combinations including EC393D and PM147D, EC393D and KE393D, or PA68D and EF165D (data not shown). Similar results were obtained using a three-detector-probe cocktail (EC393D, PM147D, and KE393D) and a five-detector-probe cocktail (EC393D, PM147D, KE393D, PA68D, and EF165D), showing no significant reduction in electrochemical signal output compared with experiments with single detector probes (data not shown).

Figure 6. Effects of a mixture of detector probes on electrochemical signal intensity. Lysates containing 16S rRNA from either *Enterococcus* or *E. coli* were hybridized with detector probes specific for *Enterococcus*, *E. coli*, or a mixture of both detector probes. In each experiment, the detector probe-16S rRNA hybrids were applied to electrochemical sensors functionalized with an *Enterococcus*-specific capture probe. Mean and SD of experiments performed in duplicate are shown. Background signal was determined in negative control (NC) experiments performed with capture and detector probes but without bacterial lysate. Experiments with the *Enterococcus* lysate show that there was no significant loss of signal intensity for detection of 16S rRNA target when hybridization was performed with a mixture of detector probes. Experiments with the *E. coli* lysate show that there was also no loss of capture probe specificity using a mixture of detector probes. Similar results were obtained with other two-, three-, and five-detector probe mixtures (data not shown).

Sensor Validation Using Control Bacterial Strains

A panel of well-characterized bacterial strains from the ATCC was tested to validate the species specificity of the UTI Chip using a seven-detector-probe mixture (UNI751D, EB1141D, EC399D, PM153D, KE399D, PA74D, and EF171D). The UNI782C capture probe detected all bacterial uropathogens tested. As expected, the EB1172C capture probe was positive for all members of the family *Enterobacteriaceae* tested and negative for *P. aeruginosa* and *E. faecalis*. The KE434C capture probe was positive for all *Enterobacteriaceae* except *E. coli*. The only species-specific capture probe positive for *E. coli* was EC434C. Likewise, the PM188C, PA111C, and EF207C capture probes were specific for *P. mirabilis*, *P. aeruginosa*, and *E. faecalis*, respectively. Figure 7 shows the results when the lysate of *K. pneumoniae* strain 13883 was hybridized with the detector probe mixture and applied to the UTI Chip. As expected, *K. pneumoniae* 16S rRNA was detected by sensors using capture probes

UNI782C, EB1176C, and KE434C but not by those using capture probes EC434C, PA102C, PM187C, and EF207C.

Statistical Analysis

Variance in signal intensity measurements using the electrochemical sensor was evaluated by comparing results from duplicate experiments. As shown in Figure 8, the SD increased roughly in proportion to mean signal intensity, consistent with a lognormal distribution as reported in a previous study.¹⁶ Analysis of variance across all experiments using logarithms (base 10) of the signals indicated that variability in the log domain was independent of bacterial species but somewhat increased for small signal intensities (Table 4). An overall pooled estimate of the SD was 0.122 log units for signals less than 100 nA and 0.056 for signals greater than 100 nA. Half-normal plots of the SD of duplicate log signals again confirmed the underlying lognormal distribution. These diagnostic plots also helped identify five outlier duplicates that were subsequently noted to be associated with one sensor in one particular experiment. As a conservative measure, we used the SD for low signals in all statistical analyses, so that 0.122 (with 75 degrees of freedom) became the SE of the difference between duplicate means in each *t*-test comparing means in the log domain.

Discussion

We have developed a sensor for direct electrochemical detection of the bacteria that cause urinary tract infection. The sensor assay system relies on efficient lysis and release of target 16S rRNA molecules. Although alkaline lysis was sufficient for release of 16S rRNA from gram-negative bacteria, this approach did not work well for the *Enterococcus*. To break down the thicker cell wall of the gram-positive uropathogens, we developed a rapid two-step lysis method involving a detergent-enzyme treatment followed by alkaline lysis. The detection system involves oligonucleotide capture and detector probes that bind to ribosomal RNA present in high copy number within the bacterial cytoplasm. The biotinylated capture probe anchors the target 16S rRNA molecule to the streptavidin-coated sensor surface, whereas the fluorescein modification on the detector probe mediates binding of the anti-fluorescein-conjugated horseradish peroxidase reporter enzyme. We found that current output was highly dependent on the distance between the capture and detector probe hybridization sites and on the location of the fluorescein modification on the detector probe. To our knowledge, this is the first detailed analysis of the determinants of signal intensity of an electrochemical sensor for detection of bacterial pathogen nucleic acids.

A fundamental difference between gram-negative and -positive bacteria is the thicker peptidoglycan cell wall of gram-positive organisms. We had previously observed that treatment with sodium hydroxide is an efficient lysis method for release of 16S rRNA from gram-negative but not gram-positive uropathogens.¹⁶ This problem in the

critical first step of the electrochemical sensor assay system seriously limited its effectiveness for detection of uropathogens because gram-positive enterococci are frequent urine culture isolates. We found that pretreatment with the cell-wall active enzyme lysozyme was found to greatly enhance alkaline lysis. In this study, we demonstrate that a combination of a nondenaturing detergent (Triton X-100) with lysozyme followed by alkaline treatment (NaOH) yielded optimal electrochemical signals for detection of the *Enterococcus*. The Triton X-100 may have additional beneficial effects, such as removal of ribosomal proteins from the 16S rRNA target molecule. Other reagents, such as the denaturing detergent sodium dodecyl sulfate, were examined and found not to be useful. One problem with sodium dodecyl sulfate may be denaturation of proteins, especially the streptavidin on the sensor surface. The concentration of Triton X-100 (0.1%) was found to be important; higher Triton X-100 concentrations resulted in loss of surface tension when the whole-cell lysate was applied to the sensor surface, resulting in cross-contamination of adjacent sensors within the array. The total lysis time of 10 minutes is a significant improvement over prior reports of lysis of gram-positive organisms with lysozyme, which may take up to 1 hour of incubation time.²⁰ This two-step lysis strategy was also effective against gram-negative bacteria, indicating its potential as a universal bacterial lysis method.

The results presented here demonstrate that maximal signal intensity requires capture and detector probes without a gap between their 16S rRNA hybridization sites. In a previous study, species-specific capture and detector probes were designed with a 6-nucleotide gap between their hybridization sites.¹⁶ However, we noted that the juxtaposed hybridization sites of the capture-detector probe pair (UNI) used to detect all bacterial 16S rRNA molecules consistently yielded higher signals than the species-specific probe pairs. For this reason, we tested the hypothesis that the distance between the capture and detector probe hybridization sites on the 16S rRNA target affects electrochemical signal intensity. For gaps ≤ 6 nucleotides, there was a strong correlation between signal intensity and the number of nucleotides between the capture and detector probe hybridization sites (Figure 3). A variety of different bacterial species-specific probe pairs without a gap between the capture and detector probe hybridization sites consistently produced higher current outputs using the electrochemical sensor than those with 6-nucleotide gaps (Table 2). This finding was independent of which part of the 16S rRNA molecule was targeted by the probe pairs; capture and detector probe pairs binding to adjacent segments of helices 6, 10, 18, and 37 of the 16S rRNA molecule of *P. aeruginosa*, *P. mirabilis*, *E. coli*, and *K. pneumoniae*, respectively, all outperformed similar probe pairs that differed only in the detector probe hybridization site.

Cooperative interactions between probes hybridizing to adjacent sites on nucleic acid targets have previously been reported.^{21–24} Flow cytometric measurement of fluorescein-modified oligonucleotide probe hybridization to *E. coli* 16S rRNA revealed significant signal enhancement

Figure 8. Relationship of variability to signal intensity. The mean signal intensity and SD for each of the 156 duplicate experiments reported in this study are included in this log-log plot, after excluding seven points with 0 SD. The pattern of the vertical scatter of the duplicates, the strong relationship between SD and mean, and analysis of the logarithm of the signal (see text) support a log normal distribution.

by addition of unlabeled “helper” probes binding to adjacent sites.²¹ Using an electrochemical sensor assay similar to ours, signal intensity was enhanced using a helper oligonucleotide binding to the region between the capture and detector probe hybridization sites of rRNA from a marine dinoflagellate.²² Gaps as short as one nucleotide between the hybridization sites of the helper oligonucleotide and biotinylated capture probe reduced detection of a double-stranded PCR product using a streptavidin-coated BIAcore sensor.²³ Oligonucleotide binding to immediately adjacent sites creates a bp stacking interaction that stabilizes hybridization.

The beneficial bp stacking effect may also relate to the effect of the location of the detector probe fluorescein modification on signal intensity. Use of 3'-fluorescein-modified detector probes resulted in higher electrochemical sensor current output than the same detector probes modified at the 5' position. The effect of fluorescein modification location on signal strength could be generalized across a broad range of bacterial 16S rRNA molecules and probe hybridization sites (Table 3). For detection of enterococcal 16S rRNA, the increase in signal intensity produced by moving the fluorescein modification from the 5' to 3' position on the detector probe was additive to the effect of eliminating the interprobe hybridization gap (Figure 4A). Several explanations for the positional effect of detector probe fluorescein modification are possible. Because the 5' end of the detector probe abuts the 3' end of the capture probe, when both probes are bound to the 16S rRNA target, a 5'-fluorescein modification would sterically disrupt bp stacking between the probes. Another explanation could involve the interaction of the fluorescein with the pocket of the antibody-binding site on

Table 4. Relationship of Signal Variance to Bacterial Species and Signal Intensity

Species	SD log ₁₀ signal (no. of duplicates)	
	Signal <100 nA	Signal >100 nA
<i>E. faecalis</i>	0.106 (21)	0.0622 (12)
<i>E. coli</i>	0.121 (15)	0.0574 (26)
Other gram-negative	0.140 (22)	0.0539 (38)
Negative controls	0.115 (17)	---
Pooled results	0.122 (75)	0.0564 (76)

the anti-fluorescein Fab fragment. Depending on the depth of the pocket, the high-affinity interaction of fluorescein with the Fab fragment could require destabilization of target-probe hybridization of the 5' end of the detector probe, again disrupting the beneficial interprobe bp stacking effect. The disruption of interprobe bp stacking by the 5'-fluorescein modification of detector probe EF171D 5'F probe (no interprobe gap) may explain why the signal output using that probe is roughly equal to that using the 3'-fluorescein-modified detector probe EF165D 3'F with the 6-nucleotide interprobe gap (Figure 4).

The increases in signal intensity from eliminating the interprobe hybridization site gap and from moving the detector probe fluorescein modification to the 3' position resulted in a dramatic improvement in sensitivity of bacterial detection using the electrochemical sensor. As shown in Figure 5, the sensitivity limit for detection improved 23-fold in experiments involving serial fivefold dilutions of *E. faecalis* and *E. coli*. This result confirms that increases in current output using the electrochemical sensor translate to improved sensitivity. The shape of the curves illustrated in Figure 5 indicates that, above threshold, the log of signal intensity varies as a linear function of the log of target concentration. In other words, there is a log-log relationship between bacterial target concentration and signal intensity. Increases in bacterial concentration should yield increases in 16S rRNA target concentration, probe-target complexes, and ultimately horseradish peroxidase molecules on the sensor surface. However, the efficiency of these hybridization and binding steps would affect the relationship between bacterial target concentration and current output, as evidenced by the earlier and steeper increase obtained using the 3'-fluorescein-labeled detector probe EF171D than that slope obtained using the 5'-fluorescein-labeled detector probe EF165D in Figure 5A. Assuming known relationship constants for the same set of capture and detector probe and reagent concentrations, it should be possible to predict bacterial target concentration using the electrochemical sensor by incorporating an internal standard into the sensor array assay.

The findings reported here demonstrate the feasibility of using mixtures of detector probes as a common reagent for species-specific detection of uropathogens using an elec-

Figure 7. Results testing the specificity of the electrochemical sensor array. Lysates of nine ATCC strains were combined with a mixture of seven detector probes and applied to the surface of the 16-sensor array (top left) functionalized with seven different capture probes (see Materials and Methods for details and Table 1 for abbreviations). Negative control (NC) sensors to which no cell lysates were applied were used to measure background signal intensity. Mean and SD of log signal intensity from duplicate sensors are plotted on the vertical axes in antilog (nanoamperes) scale.

trochemical sensor array. In previous studies, detector probes were added separately to the bacterial lysate for hybridization before deposition onto the surface of individual sensors, each coated with a different capture probe.¹⁶ That is, a separate detector probe hybridization was performed for each capture probe. Because the capture probes are designed to hybridize to different areas of the 16S rRNA target, a "universal" detector probe for each of the capture probes was not possible. We examined the possibility of simplifying the detector probe hybridization step in a series of experiments measuring the effect of hybridizing the 16S rRNA target with a mixture of detector probes. As shown in Figure 6, a representative experiment involving two detector probes, mixtures of detector probes did not reduce the intensity of the positive signal. Our results also demonstrated that detector probe mixtures did not increase background signal, indicating that the sequence of the capture probe alone was adequate to ensure specificity. The use of a probe mixture greatly simplifies the detection protocol when faced with a specimen containing unknown targets because the "universal detector probe mixture" can be used for urine specimens containing unknown bacteria. The performance of a detector probe mixture is illustrated in Figure 7 in which a UTI Chip array, containing sensors coated with different capture probes, correctly identified 16S rRNA from a panel of well-characterized ATCC strains.

In conclusion, we have developed a novel electrochemical DNA biosensor for bacterial detection. A universal lysis strategy was developed, capable of releasing target nucleic acids from both gram-positive and -negative uropathogens. The effects on sensor current output of distance between capture and detector probe hybridization sites and fluorescein modification location were examined. Finally, the feasibility of target hybridization with a detector probe mixture was demonstrated. These discoveries greatly improve the sensitivity of the sensor for detection of bacterial pathogens and reduce the design complexity of the microfluidics component when the sensor array is eventually integrated into an automated device. These discoveries demonstrate the feasibility of our long-term goal, which is to develop an integrated point-of-care diagnostic system for detection and identification of bacterial uropathogens. When coupled with a microfluidics-based sample preparation module, the sensor array will serve as the critical sensing component of molecular microbiology lab-on-a-chip.

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