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Detection of Low Concentration Pathogens and Antimicrobial Resistance Genes Using Electrochemical Oligonucleotide Tags †

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Abstract: Pathogens can be detected electrochemically by measuring guanine oxidation signals generated from RNA or DNA hybridized to a biosensor working electrode. However, the associated limit of detection (LOD) is not sufficiently low for widespread clinical use. Working electrodes employing nanomaterials such as carbon nanotubes successfully reduce the LOD but nanosensors experience high variability, poor fabrication yield and high production cost. Our work presented here demonstrates a novel approach for electrochemically detecting low concentration pathogens and antimicrobial resistance genes that transfers the guanine oxidation source from naturally-occurring RNA to synthetic oligonucleotides. In our assay, signal amplification is accomplished by binding RNA from lysed microbial cells to microparticles conjugated with millions of guanine-rich oligonucleotide tags. A sandwich hybridization assay binds RNA between a screen printed carbon working electrode conjugated with recognition probes and a microparticle conjugated with electrochemical oligonucleotide tags. These tags contain a polyguanine detection sequence and an RNA capture sequence on the same oligonucleotide. Single stranded polyguanine is prefabricated into a quadruplex to enable 8-oxoguanine signals at 0.47 V. This eliminates nonspecific guanine oxidation signals from the RNA while further reducing LOD over guanine oxidation. A 70 mer capture sequence was found to be more selective and hybridized faster at room temperature than conventional 20 mer capture sequences. Particle sizes were evaluated from 100 nm to 1.5 μm in diameter, and the larger diameter particles produced a greater detection signal. A better performance was obtained by employing magnetic microparticles and magnetically separating magnetic microparticle-RNA complexes from nonspecific materials such as lysed cell constituents and cell debris that can interfere with sandwich formation and detection. The high density magnetic microparticles rested on the electrode surface causing a portion of the oligonucleotides to adsorb to the working electrode surface.

Keywords: Biosensors; pathogen detection; antimicrobial resistance; electrochemical oligonucleotide; quadruplex

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1. Introduction

Microbial pathogens can be directly measured with an electrochemical biosensor using 16S rRNA as detection targets which are highly stable and can selectively hybridize to a working electrode with a suitable recognition probe [1–3]. Guanine molecules on the RNA are typically used as the electroactive species for label-free detection because of their relatively low redox potential [4]. However, the limited quantity of guanine molecules available for detection results in LOD that is not sufficient for clinical applications. For example, RNA with 800 bases would have approximately 200 guanine molecules based

on having 25% of the nucleotides as guanine. Depending on the specific detection technique, electrode material and sample preparation process, 10^6 to 10^{10} guanine molecules would be required for detection which limits the usefulness.

2. Technologies

2.1. Electrochemically Detectable Oligonucleotide Tags

A simple method to bring low concentration pathogens and antimicrobial resistance genes to detectable levels is to bind RNA targets with microparticles conjugated with millions of guanine-rich oligonucleotides. This provides orders of magnitude more guanine molecules for detection than direct detection of guanine on RNA. Figure 1 illustrates an oligonucleotide that has a 20 mer polyguanine detection sequence and a 70 mer capture sequence [5]. By eliminating the need for PCR or isothermal amplification, longer capture sequences can be used that are more selective and hybridize faster at room temperature than conventional 20 mer sequences.

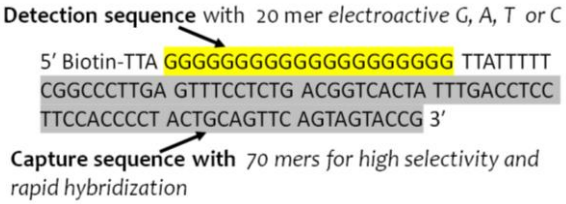

Figure 1. Oligonucleotide with 20 mer electrochemical polyguanine sequence and 70 mer capture sequence for KPC 16S rRNA.

Figure 2a illustrates how electrochemical oligonucleotide tags are used in a sandwich hybridization assay. The top layer is a magnetic microparticle conjugated with electrochemically detectable oligonucleotide tags with polyguanine and capture sequences, the middle layer is an RNA target and the bottom layer is a screen printed carbon electrode conjugated with recognition probes [5]. Each tag contains 20 guanine molecules for detection. A 1.5 μm diameter microparticle is able to bind ~10^6 biotinylated electrochemically detectable oligonucleotide tags to deliver ~20 million guanine molecules for each RNA sandwich. As noted in Table 1, approximately 5% or ~1,000,000 guanine molecules are sufficiently close to the electrode surface where guanine can contribute to the oxidation peak either directly or with an electron transport mediator. In addition ~1% or 200,000 guanine molecules are directly below the high density microparticle causing the underlying guanine molecules to adsorb to the electrode surface as illustrated in Figure 2b. Because of their proximity to the electrode, the same concentration of adsorbed guanine molecules should have a stronger signal than guanine molecules bound near the electrode surface.

Table 1. Guanine molecules available for detection per RNA target.

	RNA that is Label-Free	**RNA Bound to 1.5 μm Magnetic Microparticle with Electrochemical Polyguanine Tags**
Guanine molecules available for detection	200 guanine near electrode surface	~1,000,000 guanine near electrode surface **and** ~200,000 guanine adsorbed to electrode surface

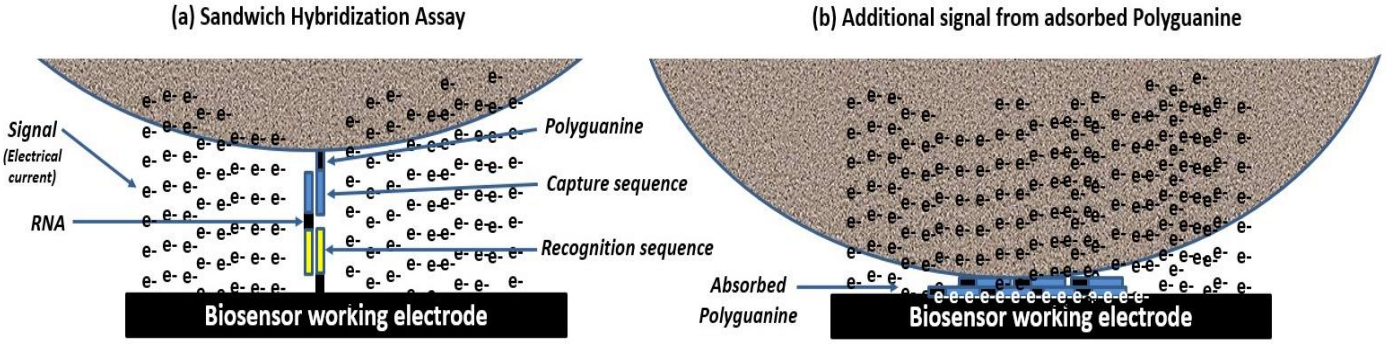

Figure 2. (**a**) Sandwich hybridization assay with magnetic microparticle conjugated with millions of electrochemically detectable oligonucleotide tags. (**b**) Sandwich hybridization assay gains additional electrochemical signal from oligonucleotide tags adsorbing to the working electrode when the high density magnetic microparticle drops onto the electrode surface.

2.2. Magnetic Particle Conjugates

Magnetic microparticles performed better than nanoparticles or nonmagnetic microparticle because of their higher surface area for binding oligonucleotides, greater contact on working electrodes for adsorbing guanine molecules from oligonucleotide tags, and better response to magnetic fields for separating conjugates from nonspecific materials. Table 2 indicates the relative signal achieved from 10,000 targets with the protocol described below using different microparticle sizes [6]. Larger microparticles with more tags generate a greater electrical current peak. Polyguanine oligonucleotides that were prefabricated into quadruplexes produced an 8-oxoguanine oxidation peak at 0.47 V relative to a baseline signal that was greater than the guanine oxidation peak at 0.90 V from single stranded oligonucleotides [7]. This allows lower LOD to be achieved and also eliminates nonspecific guanine oxidation signals from RNA.

Table 2. Relative 8-oxoguanine signal from 10^4 targets/mL from different sized magnetic microparticles.

Magnetic Particle Diameter	8-Oxoguanine Signal from 10^4 Targets/mL
500 nm	0.4 μA
750 nm	0.6 μA
1.5 μm	1.2 μA
Can go up to 20 μm	>1.2 μA

2.3. Filter Concentration and Magnetic Separation

Filter concentration with lysis and magnetic separation provide 2 logs more targets for detection as noted in Table 3 [6,8,9]. A large number of detection targets is needed to more reliably achieve low LOD particularly when targets in samples are heterogeneous and can clump to nonspecific materials. Sample volumes, filter pore size, lysis solution and the magnetic separation protocol were optimized to attain a capture yield of ~90%.

Table 3. Estimate of detection targets available from direct detection and filter concentration with magnetic separation.

	Targets in 50 μL Sample @1000 Targets/mL	Targets in Filter Concentrated 20 mL Sample @1000 Targets/mL with 90% Yield from Magnetic Separation
No. of Targets	50	18,000

3. Materials and Methods

The study tested our approach for the presence of *Klebsiella pneumoniae Carbapenemase* (KPC)-producing bacteria by detecting KPC 16S rRNA [6]. 28 samples were evaluated comprising 22 KPC-producing samples with *Klebsiella pneumoniae* ATCC BAA 1705, *Klebsiella pneumoniae* ATCC BAA-2814, or *E. coli* ATCC BAA 2340, 4 non-KPC-producing samples with *Klebsiella pneumoniae* ATCC 13883, and 2 samples with no bacteria. Bacteria were prepared in tryptic soy agar medium (Becton, Dickinson (BD), Franklin Lakes, NJ, USA) and serial diluted in broth medium and commercial urine (Sigma-Aldrich, St. Louis, MO, USA) to 10^4 cfu/mL. 1 mL samples were concentrated with a 0.45 μm filter (Pall, Port Washington, NY, USA) and re-suspended in biology-grade water and incubated at room temperature for 15 min. Bacteria were then lysed in 200 μL lysis buffer consisting of 2 M guanidinium thiocyanate (GTC), 80 mM beta-mercaptoethanol (BME), 25 mM sodium citrate, 20 μg/mL of glycogen (pH 6) with 5 μL dimethyl sulfoxide (1%) then incubated at room temperature (RT) for 5 min. The solution was mixed for 5 min with 7 μL of 1.5 μm streptavidin-coated magnetic microparticles (Bangs Laboratories, Fishers, IN, USA) conjugated with biotinylated electrochemical oligonucleotides from Figure 1 (Integrated DNA Technologies (IDT), San Jose, CA, USA) that were prefabricated into guanine-quadruplexes, then incubated at RT for 10 min. Samples were placed in a magnetic separation microtiter (Epigentek, Farmingdale, NY, USA) and a magnetic field was applied for 2 min, then the supernatant was discarded. The magnet field was removed and the magnetic particle complexes were washed with 100 μL 80 mM sodium acetate (pH 9). The magnetic particle complexes were then re-suspended in sodium acetate and allowed to hybridize for 10 min at RT on a streptavidin-coated carbon working electrode (DropSens, Asturias, Spain) conjugated with capture probes and enable sandwich structures to form. A potentiostat (PalmSens, Houten, The Netherlands) was connected and a square wave voltammetry scan produced a peak current at ~0.47 V from 8-oxoguanine oxidation in sodium acetate (pH 9). The net 8-oxoguanine oxidation signal was determined to be the difference between the 8-oxoguanine oxidation signal in sodium acetate and a baseline sodium acetate signal. The study protocol took 45 min.

4. Results and Discussion

Results are summarized on Table 4. All 22 KPC-producing samples generated a positive signal. The detection threshold of 0.2 μA was determined from the baseline sodium acetate signal variability plus 3 standard deviations. Of the 6 non KPC-producing samples, 5 reported true negative with signals below the threshold. One false negative was experienced which was possibly caused by poor sensor calibration.

Table 4. Summary of samples tested for *Klebsiella pneumoniae carbapenemase* (KPC) RNA.

	True Outcome	**False Outcomes**	
Positive test prediction	22	1	PPV = 22/23
Negative test prediction	0	5	NPV = 5/5
	TP = 22/22	TN = 5/6	

Abbreviations: TP = True Positive Rate, TN = True Negative Rate, PPV = Predictive Positive Value, PNV = Predictive Negative Value.

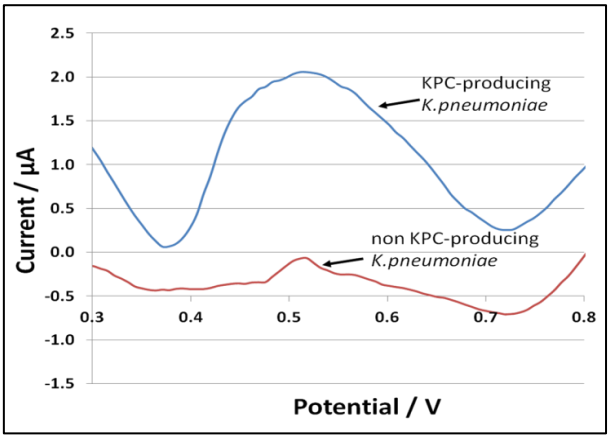

Figure 3. 8-oxoguanine oxidation peaks from positive KPC-producing *K. pneumoniae* (upper curve) and negative non-KPC-producing *K. pneumoniae* (lower curve).

The study was the first use of the electrochemically detectable oligonucleotide tags in a sandwich hybridization assay. The study was conducted with urine samples but the protocol can used for other sample types such as whole blood by adjusting the sample preparation steps for lysing, filtration, and magnetic separation.

The concentrations used in the study were 10,000 targets/mL. Positive samples were also attained from 100 targets/mL concentrations. Limit of detection will be improved by using sample volumes greater than 1 mL and microparticle diameters larger than 1.5 µm. A previous version of the technology tested water samples and filter concentrated 10 L of potable water [8].

The platform has the added capability of multiplexing a large number of targets. Multiplexing is planned for detecting 32 different targets from a sample. Each individual target will have a microparticle conjugate and 8 sensor working electrodes will be conjugated with a blend of 4 recognition probes. Working electrodes will be capable of detecting 4 different targets bound with either polyG, polyA, polyT or polyC which form oxidation peaks at different redox potentials.

Electrochemical detection with oligonucleotide tags is ideally suited for the rapid, mobile detection of complex infections that are caused by a large number of different pathogens and can require different treatments based on antimicrobial resistance. For example, sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. There are 1,700,000 US sepsis cases/yr (30–50 million global), and 350,000 US sepsis deaths/yr (11 million global) [10]. Prior to COVID-19, sepsis accounted for more than 50% of hospital deaths, and mortality increased dramatically with greater disease severity: 10–40% for sepsis, and 40–80% for septic shock [11–13]. Septic patients also represent a disproportionately high burden of hospital utilization. The average length of stay (LOS) for sepsis patients in US hospitals is approximately 75% greater than for most other conditions, and the mean LOS dramatically increases with sepsis severity: 4.5 days to 6.5 days for sepsis, and 16.5 days for septic shock [14–16]. 60% of sepsis patients are re-hospitalization within 12 months and result in a high incidence of permanent organ damage, cognitive impairment, and physical disability [17]. The annual financial impact of sepsis on the US healthcare system is $24 billion in medical costs plus $1.6 billion in litigation payments from misdiagnosis [18,19]. It has been estimated that if the US as a whole achieved earlier sepsis identification and evidenced-based treatment, there would be 92,000 fewer deaths annually, 1.25 million fewer hospital days annually, and reductions in hospital expenditures of over $1.5 billion [20].

Emergency and inpatient doctors need to select appropriate antibiotic therapy based on the specific pathogen type and drug résistance. At the first sign of sepsis, therapy needs to be determined within hours but blood cultures take 3 to 5 days. When the patient is in

sepsis shock, each hour delay from appropriate antibiotic therapy has a 6% drop in survival [21,22]. Alternative approaches to determine pathogen type and drug resistance enzymes can employ a 12-h blood culture followed by multiplex PCR using sophisticated instruments. These approaches have a 13–17 h sample-to-result turnaround time, are too expensive for many hospitals, are too large for many emergency departments and can incur culture errors that cause false positive and false negative outcomes.

The electrochemically detectable oligonucleotide tag platform can be configured to operate in a mobile test cartridge. It has the potential to detect 32 pathogens and antibiotic resistance enzyme genes associated with sepsis in whole blood without a culture in under 1 h to greatly reduce the time to administer an appropriate antibiotic. The mobile electrochemical reader can fit in an emergency room's limited available space. In addition it can potentially be used to test patients with sepsis-related vital signs for blood infections by telehealth practitioners and physician at home specialists before patients are sent to the hospital to save valuable time.

Quantification from the signal peak amplitude is proportional to the target concentration and also be used to determine the effectiveness of a specific antibiotic by measuring the change in signal strength from a second sample incubated a few hours in different antibiotic inoculums.

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Conflicts of Interest: The authors declare no conflict of interest. Neil Gordon is the founder and president of Guanine, Inc., and has a financial interest in the company. Raj Bawa, PhD, MD, is vice president and chief IP officer of Guanine, Inc., and also serves as scientific advisor to Teva Pharmaceutical Industries, Ltd., Israel.

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