Simple, Fast and Highly Sensitive Detection of Gram-Negative Bacteria by a Novel Electrical Biosensor

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***Abstract*—This work presents a rapid, low-cost, highly sensitive and specific capacitive sensor for detection of Gram negative bacteria in a field setting. Recognition of Gram-negative bacteria is based on specific detection of lipopolysaccharides (LPS) by LPS-specific aptamer probe immobilized on electrode sensors. An inhomogeneous AC electric field is applied on sensor electrodes and induces positive dielectrophoresis that attracts LPS particles to the sensor electrodes for rapid detection. The same AC signal is also used to detect the binding reactions occurred on the sensor surface. The AC signal was optimized, and the binding between LPS and the specific aptamer was demonstrated. The detection limit reaches as low as 4.9 fg/mL for free LPS molecules and 53 #/mL of bacteria within a 30s' response time, meeting the needs of on-site bacteria detection.**

I. Introduction

Currently, on-site or point-of-care detection of pathogens and infectious diseases has unmet needs of finding a rapid, sensitive, specific and simple-to-use sensing method. Rapid, sensitive and reliable detection of pathogenic bacteria is critical to diagnose and differentiate infectious and bacterial diseases.

Gram-negative bacteria is one of the most common pathogenic microorganisms, such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, and other lesser known pathogens. Its effective detection and recognition from Gram-positive bacteria or other microorganisms has been attracting much attention over the years [1, 2]. Lipopolysaccharide (LPS), also known as lipoglycans and endotoxins, is the major component of the outer membrane of Gram-negative bacteria. Since LPS is a distinct molecular pattern that is only found on Gram-negative bacteria, its detection is pivotal for food safety, pathogen identification and differentiation as well as a guide to proper clinical treatment.

Conventional methods of bacterial detection rely upon cell culture, microscopic analysis, and biochemical assays. These procedures are time-consuming, costly, and require special equipment and skilled users. In contrast, biosensors can be a good approach to achieve field deployable bacterial detection. Extensive research has been done on bacteria detection. Among label-free bacteria whole-cell sensors, the lowest limit of detection (LOD) reported was 10^3 cells/mL with response time from tens of minutes to hours [3].

This work presents a rapid, highly sensitive, low-cost and specific aptasensor for label-free detection of Gram-negative bacteria. Recently, we have developed a sensitive and specific capacitive sensing technology, "alternating current electro-kinetics (ACEK) capacitive sensing"[4, 5], which can detect specific binding rapidly (<1 minute from sample to result) by a lay person with a single-step, no wash operation. We have success with a series of proof-of-concept experiments detecting specific antibodies, protein biomarker, virus and bacteria from clinical samples, small molecules [6, 7], and nucleic acid [8] from simulated samples. Currently we have femtomolar (fM) LOD for antibody and small molecules in phosphate-buffered saline (PBS) based solution, and we successfully detected disease-specific antibodies from serum and virus particle from nasal swab sample with less than 60-second response time [9].

In this work, this method has been adapted to detect Gram-negative bacteria. Using microelectrodes functionalized with LPS-specific aptamer probes and combining with the suitable AC signal, sensitive and specific detection can be achieved for two types of target molecules, free LPS molecules and *Escherichia coli (E. coli)* as a model of Gram negative bacteria. The LODs are 4.9 fg/mL for free LPS molecules and ~100 #/mL for *E.coli*. Further, *E.coli* can be quantitatively detected without suffering from the interference caused by the free LPS in the growth medium. The "sample to result" response time is within 30 sec.

II. Mechanisms

In this work, aptasensors are prepared by immobilizing LPS specific aptamer on interdigitated microelectrodes. An

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inhomogeneous AC electric field is applied on the sensor and induces positive dielectrophoresis (pDEP) that attracts LPS particles to the sensor electrodes. The binding is detected by the same ACEK signal by measuring the change in interfacial capacitance (C_{int}) at the surface of micro-electrodes, as shown in Fig. 1. It is a single button process.

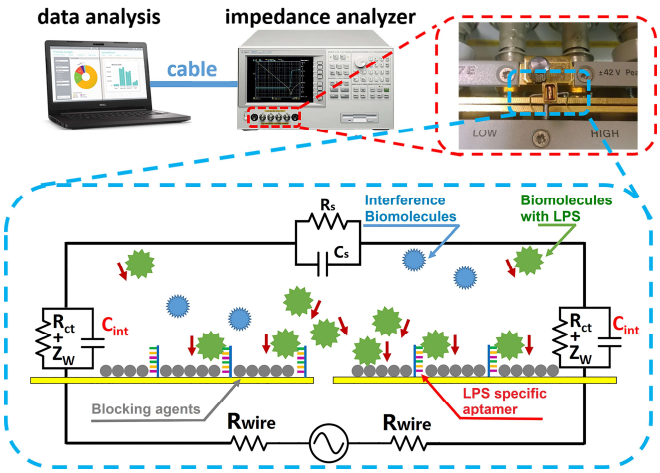

Figure 1 Schematic of detection mechanism. It is based on measuring the interfacial capacitance (Cint) on functionalized microelectrodes with an AC signal capable of inducing positive DEP, which attracts the target molecules to the sensor.

A. Dielectrophoresis Enrichment

The short sample-to-result time of our diagnostic system is achieved by incorporating ACEK effect into capacitive sensing. With appropriate AC signals and microelectrodes, the detection protocol will allow the induction of ACEK effects that will lead to microflows around the electrodes and bioparticle movement toward the electrodes. As LPS and bacteria are the analytes of interest here, the dominant enrichment mechanism is DEP due to their sizes. Within an inhomogeneous electric field, DEP can attract or repel particles based on the difference between the particle polarizability and that of the medium solution. The DEP force on a spherical particle can be expressed as

$$F_{DEP} = \pi\varepsilon_m a^3 \, \mathrm{Re}\left[\frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*}\right] \nabla|E|^2 \qquad (1)$$

where ε_m is the medium permitivity, a is the particle radiater and E is electric field strength. ε_p^*, ε_m^* are the complex permittivities of particle and medium , respectively. Complex permittivity is defined as $\varepsilon^* = \varepsilon - j\frac{\sigma}{\omega\varepsilon}$, where ω is angular frequency and j is the imaginary unit, defined as $\sqrt{-1}$. Because complex permittivities are frequency dependent, the DEP force is also a function of AC frequency.

According to Eq. (1), F_{DEP} is proportional to r^3. The bacteria we detected is in the scale of micron meters (*E. coli* is about 0.5 μm in diameter and 2 μm long). As a result, FDEP should be the dominated force against other AC electrokinetic effects. In bacteria samples, there may be free LPS molecules of nanoscale (~10 nm), which are much smaller than bacteria cells. By using a low AC voltage, F_{DEP} on LPS can be negligible, thus avoiding false positive detection by free LPS molecules. As a result, the interference from free LPS molecules can be safely excluded.

B. Capacitive Immunoassay

This capacitive sensor detects specific protein binding by measuring the interfacial capacitance at the surface of electrodes. As bacteria continues binding onto the electrodes, the thickness of interfacial layer keeps increasing which leads to a decrease of the interfacial capacitance, C_{int}. C_{int} is monitored during the detection, and its change rate is adopted as the measurant. By monitoring the change rate of C_{int}, specific binding of target analytes can be detected and quantified. The benefit is two folds. (1) The readout data will reflect only the molecule accumulation on the electrode surfaces, effectively removing the effects of interfering constituents in test solutions, hence no wash step is needed; and (2) no reference cell is needed since this method monitors the dynamic binding process, further simplifying the instrumentation.

Biomolecular deposition at electrode surface would change the value of interfacial capacitance (C_{int}) as follows.

$$C_{int} = A_{int}/\left(d_p/\varepsilon_p + d_{edl}/\varepsilon_s\right) \qquad (2)$$

where ε_s and ε_p are permittivity of the solution and bioparticle, A_{int} is the surface area of the interfacial capacitor of the functionalized electrode, d_p and d_{edl} are the thickness of biomolecular deposition and electric double layer formed on the electrodes surface respectively.

III. Experimental Details

A. Interdigitated Electrode Biosensor

Gold thin film interdigitated electrodes were used for quantifying the bacteria concentration. The microelectrodes consisted of 100 nm gold (Au) over 5 nm chromium (Cr) on oxidized (100 nm SiO^2) silicon wafer. The Au/Cr metallization was deposited by electron beam evaporation and patterned by lift-off process. The electrodes are 2 μm wide with 2 μm separation in between.

The bare electrodes were washed with acetone, isopropyl alcohol, and de-ionized water sequentially, to remove possible organic contamination on the surface. Following the cleaning procedure, the bare electrodes were checked with microscope and tested with an impedance analyzer. The capacitance values of clean electrodes were as a quality control step of the sensor. The average capacitance value for cleaned SAW chip was ~7 nF at 10 kHz. The capacitance variations for the bare electrode are very small, less than 0.07%. With this capacitance value, the sensor surface is considered as adequately clean and ready for the next step.

B. Reagents and Sensor Functionalization

All chemical reagents were purchased from ThermoFisher (Grand Island, NY). The buffers used in the detection were based on PBS. 0.1×PBS (Phosphate-buffered saline) was prepared by 1:10 volume dilution of physiological strength PBS (i.e. 1x PBS) in ultrapure water to obtain 0.5 mM phosphate buffer (pH 7.0) containing 7.5 mM sodium chloride.

Sensor functionalization includes immobilization of cocaine-specific probe and blocking the surface of the electrodes. The immobilization of the probe is performed by

loading 10 µL of 10 µM cocaine-specific aptamer diluted in 0.01× PBS over the electrode and incubating it in a humidor for 24 hours. 1.0 mM 6-mercaptohexanol was used to block the surface. After 3 hours of blocking, the chips are ready for testing.

C. *Testing procedure and impedance measurement*

A high precision impedance analyzer (Agilent® 4294A) was used to apply a symmetric AC signal on the sensor's electrodes continuously for 30 seconds. Raw data of C_{int} measurement was recorded simultaneously. After performing a least square linear fitting algorithm on the obtained raw data of C_{int} measurements, the percentage change rate, d|C|/dt in %/min, was obtained from the readout of the sensor, indicating the binding reaction occurring on the electrodes' surfaces.

IV. Results and Discussion

A. *Impedance Characterization*

First, the impedance spectrum of the electrode/electrolyte cell was examined to extract its electric equivalent circuit, and to determine which elements would predominately account for the bacteria deposition at the electrode surface. Impedance between a pair of parallel electrodes immersed in liquid can be approximated as a network of capacitor and resistive components as shown in Fig.1. Of the four different circuit elements, R_{ct} and C_{int} describe the electrical properties at the electrode/electrolyte interface, while Rs and Cs are the resistance and capacitance of bulk solution.

A few considerations go into choosing an optimal AC frequency for our sensing technology. First, AC signal at this frequency should enhance the mass transport of biomolecules. In our experiment, the enrichment effect by ACEK has been observed to be the most pronounced at 100 kHz [4]. Therefore, 100 kHz is adopted as the testing frequency in our ACEK impedance sensing. i.e. reading the electrode impedance continuously at 100 kHz.

Second, at the testing frequency, C_{int} and Rs should dominate the current path, so the equivalent circuit can be simplified as a serial connection of C_{int} and Rs. Consequently, the measured C will be directly proportional to C_{int}. In another word, it should be relatively straightforward to extract C_{int} that account for the surface binding.

B. *Effect of Bacterial Growth Phase*

At first, Gram-negative bacteria were tested using bare electrodes, with an AC voltage of 500 mVrms at 100 kHz, and 1xPBS was used as the control sample and the diluent. Bacteria concentrations from 10^4 to 10^7 #/mL were tested for a duration of 5 minutes.

The normalized capacitance readings as a function of time were shown in Fig. 2. The measured capacitance was normalized to the capacitance value at test time 0. The normalized capacitance showed a monotonous change with time, and different bacterial concentrations showed different change rate. The change rate became more negative with increasing bacterial concentration.

Next, bacteria in two different growth phase, stationary phase and exponential phase, were tested under the same conditions. Table 1 listed the sensor responses of Alcanivorax borkumentsis for the concentrations from 10^4 to 10^7 #/mL. No difference in the sensor output could be found. Therefore, it can be concluded that bacterial growth phase will not cause appreciable changes in the sensor response.

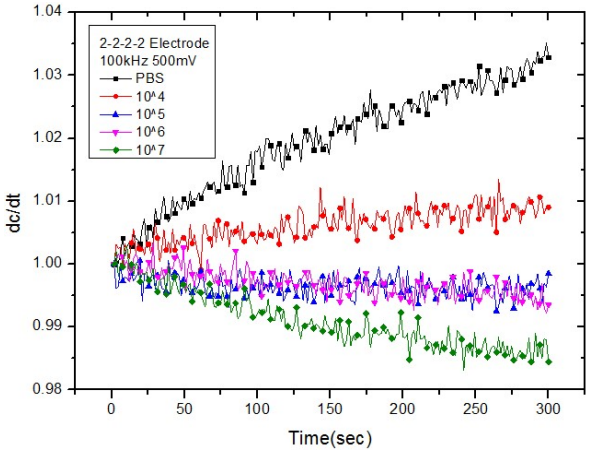

Figure 2 Normalized capacitance change for bacteria concentrations from 10^4 to 10^7 #/mL over 5 minutes.

TABLE I. Comparison of Detection Results between Exponential and Stationary Growth Phases of Bacteia

Bacterial Concentration	Detection Results (%/min)	
	Exponential Phase	***Stationary Phase***
Control	5.31	5.58
10^4 #/mL	3.65	3.71
10^5 #/mL	2.78	2.90
10^6 #/mL	-2.49	-2.15
10^7 #/mL	-5.33	-5.06

C. *Effect of AC frequency and Magnitude*

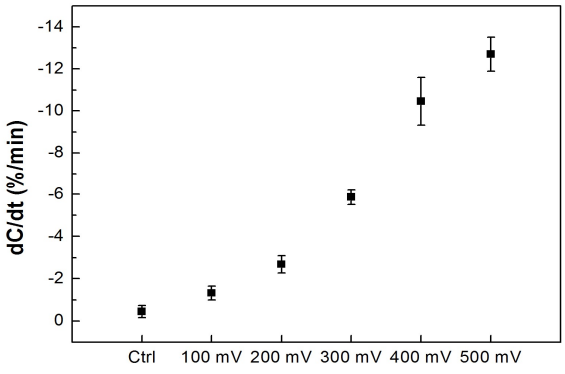

Figure 3 Capacitance change rates at AC voltages from 100 mV to 500 mV for 100 fg/mL LPS molecules in 0.1x PBS. The control is 0.1x PBS.

In a subsequent set of experiments, sensors functionalized with LPS-specific aptamer were used to detect free LPS in 0.1x PBS. The responses showed a clear dependence on the applied voltage, and also clear differentiation from that of control samples. It supports that specific binding between LPS and the aptamer probe took place and the binding events were enhanced by a stronger electric field. It can also be found that

sensor responses became very small for voltage below 100 mV. If a low voltage was used for bacteria detection, there is no concern for the interference of free LPS in the matrix [9].

As can be from Eq. 1, DEP force is frequency dependent. Therefore, several frequencies were tried in order to optimize the sensing frequency. *E.coli* sample at $6.4x10^4$ /mL was tested from 1 kHz to 60 MHz. Only 15 mV was used here, as no difference in sensor responses was observed when the applied voltage was lowered from 500 mV to 15 mV in another experiment. This makes sense because bacteria are relatively large bioparticles, and only 15 mV was needed in our previous detection of virus particles [10]. The responses were plotted in Fig. 4, and 100 kHz is clearly shown as the optimal test frequency. This also corroborates that DEP effect enriches the targets to the sensor, since DEP force is frequency-dependent.

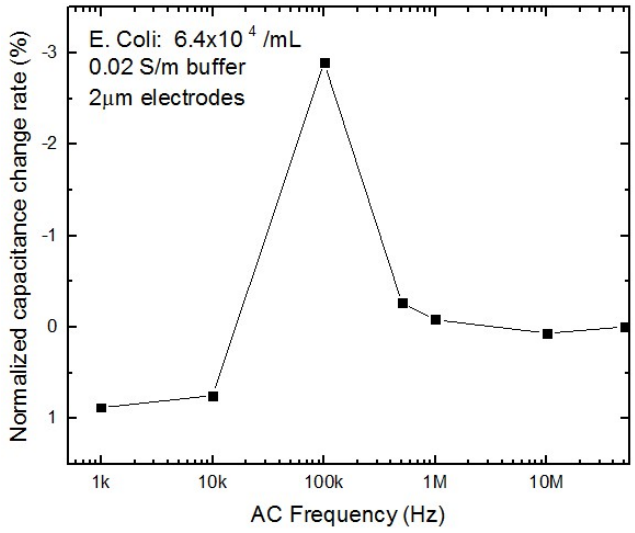

Figure 4 Capacitance change rates for AC frequencies of 1 kHz, 10 kHz, 100 kHz, 300 kHz, 1 MHz, 10 MHz and 60 MHz. 15 mV was used for testing.

D. Dose Response of Bacteria Detection

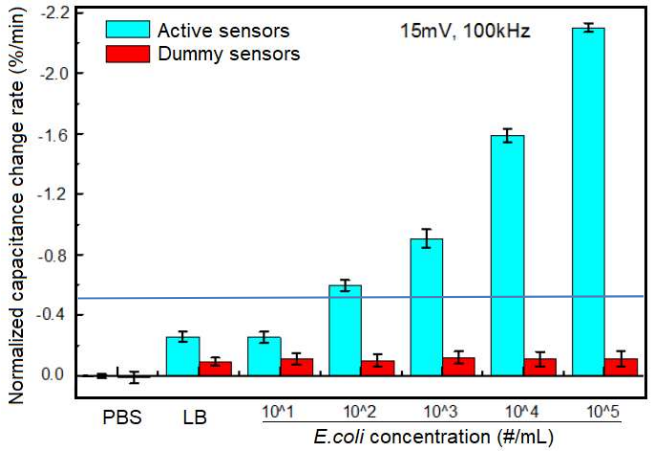

Figure 5 Dose response of *E. coli* as a function of concentration. The cut-off line is -0.507 %/min, and the detection limit is 53.1 #/mL.

Figure 5 shows the normalized capacitance change rates (dC/dt) for 10^2 to 10^5 #/mL of *E. coli* as a model of Gram-negative bacteria. The responses of aptamer functionalized sensors and dummy sensors are compared. For control tests, 0.1x PBS and Lysogeny broth (LB) growth medium were also tested and presented here. The LB medium was used to grow bacteria and contained free LPS.

There were very little responses from the dummy electrodes and the control samples, attesting to the specificity of this bacteria sensor. A linear, inverse correlation between dC/dt and *E. coli* concentration can be observed in Fig. 5. The dependence is expressed as y(%/min)= $0.364 - 0.505\,lgx$ (bacteria concentration in #/mL), with Pearson correlation coefficient R^2=0.981. The LOD is defined as 3 standard deviations from the response of background control (LB: -0.324 ± 0.061%/min). The cut-off dC/dt is calculated to be -0.507 %/min, which corresponds to an LOD being 53.1 #/mL for *E. Coli*.

V. Conclusion

In summary, we developed a capacitive aptasensor based on DEP enriching effect for rapid and label-free detection of viable Gram-negative bacteria. An LOD of 53.1 #/mL was obtained for Gram-negative bacteria, which is of a high sensitivity compared with existing reports. The sensor is also rapid with 30 s response time. Further development of this work will lead to a range of simple, sample-in-result-out sensing technologies that will be considerably more sensitive and significantly faster than traditional immunoassay (e.g. ELISA) methods. It is also anticipated that the sensing method would be applicable to numerous diagnostic applications including infectious diseases, metabolic diseases, etc.

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