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Short communication

A methodology for rapid detection of *Salmonella typhimurium* using label-free electrochemical impedance spectroscopy

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ABSTRACT

A pathogen detection methodology based on *Bayesian decision theory* has been developed for rapid and reliable detection of *Salmonella typhimurium*. The methodology exploits principles from statistical signal processing along with impedance spectroscopy in order to analytically determine the existence of pathogens in the target solution. The proposed technique is validated using a cost-effective and portable immunosensor. This device uses label-free, electrochemical impedance spectroscopy for pathogen detection and has been demonstrated to reliably detect pre-infectious levels of pathogen in sample solutions. The detection process does not entail any pathogen enrichment procedures. The results using the proposed technique indicate a detection time of approximately 6 min (5 min for data acquisition, 1 min for analysis) for pathogen concentrations in the order of 500 CFU/ml. The detection methodology presented here has demonstrated high accuracy and can be generalized for the detection of other pathogens with healthcare, food, and environmental implications. Furthermore, the technique has a low computational complexity and uses a minimal data-set (only 30 data-samples) for data analysis. Hence, it is ideal for use in hand-held pathogen detectors.

1. Introduction

Food-borne illnesses are an increasing cause of concern around the world. These infectious diseases cause distressing, and sometimes extreme health problems. Most of these diseases are caused by pathogens, predominantly—bacteria. One such microbe is *Salmonella enterica* serovar Typhimurium. It is a Gram-negative pathogen that is generally transmitted to humans through the consumption of animal-related products such as egg, meat, or milk. Infections are also caused by contact with contaminated fruits, vegetables, water, or livestock. This pathogen is known to cause a wide variety of diseases such as *Salmonellosis*, typhoid fever, food-poisoning, gastroenteritis, and septicemia. These diseases can be potentially fatal if timely treatment is not provided. According to the Center for Disease Control and Prevention (CDC), nearly 40,000 cases of *Salmonellosis* are annually recorded in US alone (CDC, 2006). The National Institute of Health (NIH) reported *Salmonella* as the most common cause of food-borne illness. Hence, the risk of widespread infection of *Salmonella* needs to be urgently addressed. This possibility of *Salmonella* contamination of water and food sources also makes it a potentially grave bioterrorism threat. There have been such cases involving *Salmonella* in the past (Tucker, 1999). The CDC has listed *Salmonella* as a Category B bioterrorism agent. The economic implications of *Salmonella* are also significant. For 2006, the United States Department of Agriculture estimated the cost of *Salmonella* related illnesses to be close to $2.5 billion, which included $191 million in medical care and $92 million in lost productivity (ERS, 2007). The World Health Organization has also expressed concerns about the public health burden in developing countries due to *Salmonella* (WHO, 2002). Hence, there is a need to develop rapid and reliable sensors to detect the presence of this pathogen. A detection system that can detect pathogens at pre-infectious levels has the potential to be a valuable asset for early mitigation of possible food- and water-borne diseases and illnesses.

Biosensors have emerged as extremely useful tools for pathogen detection. The reaction between the analyte and the corresponding

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recognition molecules conjugated to the biosensor is captured by the biosensor's transducer element to provide measurable electrical signals. A comprehensive survey of techniques analyzing these techniques is available (Mohanty and Kougianos, 2006). Extensive research has been done on biosensing and a wide array of biosensors has been developed for detection of a broad range of pathogens and pathogen concentrations (Yang et al., 1998, 2003, 2004a,b; Andresen et al., 2002; Radke and Alocilja, 2004, 2005; Oh et al., 2004; Rijal et al., 2005; Su and Li, 2005; Subramanian et al., 2006). However, many of these techniques are either time-consuming, or require secondary and tertiary labels, or highly skilled technical labor, or work only with large pathogen concentrations. Impedance-based methods have been widely regarded as a rapid means for pathogen detection (Silley and Forsythe, 1996). Electrochemical biosensors are highly sensitive, rapid and inexpensive (Ghindilis et al., 1998). Among the biosensor platforms, label-free electrochemical impedance spectroscopy (EIS) has been reported to be a good technique to investigate bio-molecular interactions (Katz and Willner, 2003; Davis et al., 2005; Tlili et al., 2006) due to its simplicity.

In this work, we present a simple but efficient methodology to rapidly detect *S. typhimurium*. The proposed methodology involves the measurement of the system impedance of an electrochemical cell over time after providing a small-amplitude sinusoidal signal excitation. We then apply principles from Bayesian decision theory in order to develop an analytical tool (referred to as a detector) that can predict the existence of the pathogen in the given sample. *Bayesian* detection principles are used for computation since such a detector has been proven to have minimum chance of error in its operation (Kay, 1993).

2. Experimental details

2.1. Bacterial culture and sensor details

A culture of *S. typhimurium* was grown overnight at 37 °C in Lennox broth (L.B); inoculated in fresh L.B at a dilution of 1:200 and subsequently allowed to grow for 10–12 h at the same temperature. The initial pathogen concentration in the sample was obtained by performing live cell counts using a haemocytometer and an optical microscope. The required pathogen concentration of ~500 CFU/ml was obtained after appropriate dilutions and verified using optical density (OD) measurements at a wavelength of 600 nm. The detailed techniques and results provided in the supplemental material section are available online.

The biosensor used for the experiments was a standard three-electrode sensor comprising of an Ag/AgCl reference electrode, a gold working electrode, and a gold counter electrode developed on a piece of printed circuit board (PCB). Each PCB sensor chip is a single-sided copper clad board with positive photo-resist on a fiberglass substrate. Details of the sensor fabrication are described in (La Belle et al., 2007). *S. typhimurium* specific antibodies were coated onto the biosensor's working electrode to bind to the bacteria.

2.2. Determination of optimal excitation frequency for S. typhimurium

In order to determine the frequency at which the maximum changes in impedance occur for *S. typhimurium*, the impedance of a set of target samples were recorded for a range of frequencies (1–100,000 Hz) using an CHI660C Electrochemical Workstation (CH Instruments). The alternating current (AC) impedance setting was set to amplitude of 5 mV with a reference potential of 250 mV. A plot was then made for frequency versus the maximal% change in impedance for each test sample. This was done to determine an average frequency that could be used for comparison between different PCB electrodes, controls, and samples. These plots (of frequency versus impedance at various concentrations of target) were used to compute the frequency of maximal response. More details on this procedure for the optimal frequency selection are provided in the supplemental material.

2.3. Impedance measurements

For all impedance-related experiments, we used the impedance spectrometer (CHI 660C). The instrument has the capability to provide the excitation signals and also record the system impedance that arises due to the binding events occurring at the surface of the working electrode. A small AC potential (signal details provided below) was given to the device via the counter electrode (C) and the output potential was measured between the working (W) and the reference (R) electrodes.

Before running impedance measurements, each biosensor chip was rinsed with freshly prepared redox-probe solution. The purpose of using the redox-probe is to act as a mediator to enhance electron transfer. It is activated by applying the initial reference potential. The redox-probe solution was prepared using PBS buffer (pH 7.4) and 5 mM potassium ferrocyanide/ferricyanide. The test analytes containing the required pathogen concentrations were obtained by diluting the pathogen sample with the redox-probe. All the pathogen concentrations used for testing were lower than the infectious level (1000 CFU/ml) and were stored at 4 °C until used. At the time of the experiment, the analyte sample was pipetted into the well of the PCB chip and the AC excitation was set to amplitude of 5 mV, at the optimal excitation frequency, and with a reference potential of 250 mV. The system impedance was recorded for duration of 5 min. The impedance was measured every 10 s to obtain a total of 30 data-samples over the 5-min measurement interval. The 5-min duration was chosen to ensure that the live-pathogen concentration remained unchanged over the measurement interval.

The experiments were performed for five different biosensor chips chosen from different lots, in order to test the robustness of the methodology. The experiment for every chip consisted of two runs—one with a clean solution and the second with an equal volume of infected solution.

2.4. Statistical analysis

In order to test for the existence of pathogens in the analyte, we apply Bayesian detection principles from statistical signal processing. The pathogen detection problem is formulated as a binary hypothesis problem as follows:

H_0. Clean analyte.

H_1. Contaminated analyte.

H_0 and H_1 are referred to as the null and alternate hypotheses, respectively. The data for the two hypotheses were derived from the impedance measurements. We obtained the best curve-fits for the impedance–time plots corresponding to the clean and infected solutions. We then statistically characterized the parameters of each fit. These parameters constituted the data for each hypothesis. Subsequently, we developed a Bayesian detector (Kay, 1993) using the fitting parameters as random variables and used this as a metric to predict the existence of pathogens in the solution. A Bayesian detector was used since it minimized the probability of error for a given test parameter (Kay, 1993). The software package Matlab was used for all our analysis.

The method described here is a novel and rapid means of comparing hypotheses with large data-sets that are statistically

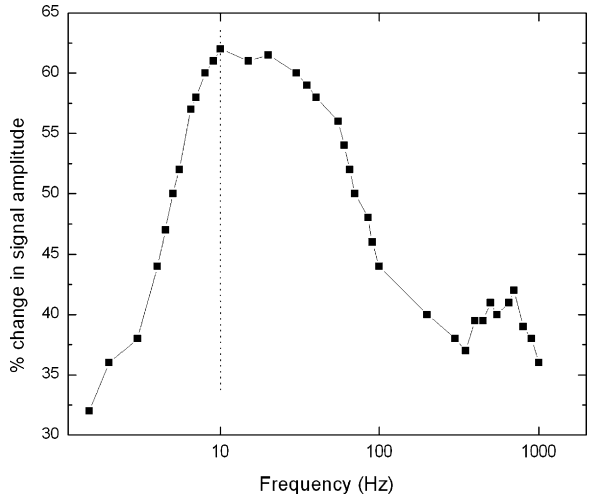

Fig. 1. Maximum change in signal (corresponding to the response of *S. typhimurium*) is observed at 10 Hz.

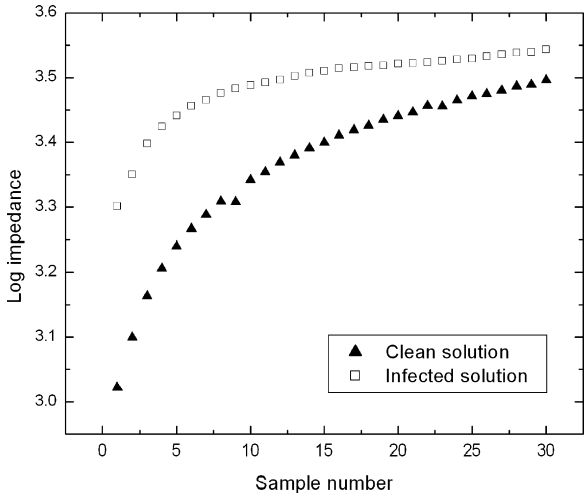

Fig. 2. Logarithmic impedance curves for clean and infected solutions (volume of 100 μl) over time (represented by discrete sample numbers). The samples represent impedance values at time $T = 10 \times$ (sample number) seconds. The difference between the clean and infected solutions is significant.

incompatible with known probability distribution functions. It is not computationally intensive and has a worst case execution time of the order of $O(n^3)$, where 'n' is the number of fitting parameters (in our case, $n = 3$). Since the technique utilizes only 30 data-samples for analysis, it is also memory-efficient. Hence, the proposed methodology can easily be incorporated in hand-held detectors for application in real-time situations.

3. Results

3.1. Optimal excitation frequency for maximal response from S. typhimurium

The maximal response of *S. typhimurium* was found to occur at a frequency of ~10 Hz (Fig. 1). Hence, 10 Hz can be used as a reliable excitation frequency while testing for *S. typhimurium*.

3.2. Impedance measurements over time

As mentioned earlier, the impedance measurements for each chip were recorded first for a clean solution and then for a solution containing *S. typhimurium* (concentration ~500 CFU/ml). The data was then plotted for the different chips. A typical plot for one chip is shown in Fig. 2. We observed a steady and significant increase in impedance in the case of a pathogen containing solution over the same volume of clean solution. The experiment was repeated with five different chips and we observed a similarity in all the plots (not shown for brevity). The similarity in the plots proved the uniformity in the sensor's response. Additionally, the similarity in the outputs of sensors fabricated at different times indicated the device's stability in spite of process variations.

3.3. Analysis of impedance data

The analyses were performed using Pentium-4, 2 GHz computer, and all the computations were accomplished within 1 min. The closest curve-fit for the plots was determined to be an exponential fit, of the form $ae^{-bx} + c$. The average R^2 value for the fits was approximately 0.99. The fitting parameters (a, b and c) were obtained using Matlab. The parameter sets corresponding to the curves shown in Fig. 2 were found to have Gaussian distributions (statistics listed in supplement). We used this data to develop the Bayesian detector for this problem. For applying Bayesian detection, we need to know prior probabilities for the two hypotheses (Kay, 1993). In a real-time pathogen detection scenario, we would not have any idea about the existence of pathogens in the solution. Hence, it is reasonable to choose equal prior probabilities ($p = 0.5$). These values were used along with the parameter statistics (listed in supplement) to calculate the decision metric. The analytical procedures associated with the detector computation are detailed in the supplemental material.

Table 1
Results of statistical analysis

Case 1: Chip 1: target is contaminated	
Detection threshold (γ) (normalized)	1.7431
Value computed using Λ (normalized)	1.7495
Result ($\Lambda > \gamma$)	Yes
Interpretation	Solution is contaminated
Case 2: Chip 2: target is clean	
Detection threshold (γ) (normalized)	−1.095
Value computed using Λ (normalized)	−1.095
Result ($\Lambda > \gamma$)	No
Interpretation	Solution is clean

Sample test results that were obtained after applying the derived decision rule on different target sample analyte parameters are shown in Table 1. The performance of the detector were evaluated for the worst case scenario (*i.e.*, with test input value for detector equal to the difference between the infected solution parameter and the standard deviation of that parameter). In case 1, we tested for a contaminated solution and in case 2, we tested for a clean solution. We observed that our proposed detection technique successfully predicts the nature of the target analyte in both cases. The tests were performed on four other chips (each from a different lot) with contaminated analytes, and we were able to successfully predict the nature of the target in all those cases. The successful detection showed that our technique was robust over the effects of process variations.

4. Conclusions

We have demonstrated a novel, rapid detection methodology using label-free, cost-effective, and reproducible EIS-based sensors. With this Bayesian detection technique we were able to successfully

detect *S. typhimurium* concentrations in the order of 500 CFU/ml, which is lower than infectious pathogen levels. The accuracy of the proposed detection methodology has been demonstrated on positive and negative target scenarios. This detection technique can also be generalized for the detection of other pathogens, provided specific antibodies are available for them. The technique has a low computational complexity (proportional to the number of fitting parameters) and is able to provide the correct result using just 30 data-samples. Hence it is ideally suited for application in hand-held detectors. This rapid, pre-infectious level pathogen detection mechanism could greatly benefit the food and health industries, and national security, among others.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.06.036.

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