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Nano-particle enhanced impedimetric biosensor for detection of foodborne pathogens

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Abstract. Recent outbreaks of foodborne illness have been increased the need for rapid and sensitive methods for detection of these pathogens. Conventional methods for pathogens detection and identification involve prolonged multiple enrichment steps. Even though some immunological rapid assays are available, these assays still need enrichment steps result in delayed detection. Biosensors have shown great potential for rapid detection of foodborne pathogens. They are capable of direct monitoring the antigen-antibody reactions in real time. Among the biosensors, impedimetric biosensors have been widely adapted as an analysis tool for the study of various biological binding reactions because of their high sensitivity and reagentless operation. In this study a nanoparticle-enhanced impedimetric biosensor for *Salmonella* enteritidis detection was developed which detected impedance changes caused by the attachment of the cells to the anti-*Salmonella* antibodies immobilized on interdigitated gold electrodes. Successive immobilization of neutravidin followed by anti-*Salmonella* antibodies was performed to the sensing area to create a biological detection surface. To enhance the impedance responses generated by antigen-antibody reactions, anti-*Salmonella* antibody conjugated nanoparticles were introduced on the sensing area. Using a portable impedance analyzer, the impedance across the interdigital electrodes was measured after the series of antigen-antibody bindings. Bacteria cells present in solution attached to capture antibodies and became tethered to the sensor surface. Attached bacteria cells changed the dielectric constant of the media between the electrodes thereby causing a change in measured impedance. Optimum input frequency was determined by analyzing frequency characteristics of the biosensor over ranges of applied frequencies from 10 Hz to 400 Hz. At 100 Hz of input frequency, the biosensor was most sensitive to the changes of the bacteria concentration and this frequency was used for the detection experiments. The biosensor was able to detect 10^6 CFU/mL in phosphate buffered saline (PBS) with a detection time of 3 minutes. Additional use of nanoparticles significantly enhanced the detection performance. By using the nanoparticles the biosensor could detect 10^4 CFU/mL of *Salmonella* enteritidis in PBS and 10^5 CFU/mL of cells in milk.

1. Introduction

Salmonella enteritidis is one of the major foodborne pathogens of concern. It is a gram-negative rod-shaped bacterium that causes severe illness in the elderly, infants, and those with weak immune

systems. A person infected with this pathogen shows symptoms of fever, abdominal pain, nausea and vomiting, diarrhea, dehydration, weakness, and loss of appetite. The symptoms may begin to appear 12 to 72 hours after consuming a contaminated food or beverage. The pathogen is usually associated with raw or undercooked eggs and poultry. *Salmonella* enteritidis outbreaks continue to occur, and *S.* enteritidis-related outbreaks from various food sources have increased public awareness of this pathogen.

Conventional methods for *Salmonella* detection and identification involve prolonged multiple enrichment steps. Even though some immunological rapid assays are available, these assays still require enrichment steps and give results in 18-48 h. Biosensors have shown great potential for rapid detection of foodborne pathogens. They are capable of direct monitoring the antigen-antibody reactions in real time. Among the biosensors, impedimetric biosensors have been widely adapted as an analysis tool for the study of various biological binding reactions because of their high sensitivity and reagentless operation. The impedimetric biosensor enables qualitative and quantitative monitoring of bacteria by measuring the changes in the electrical impedance. Electrochemical impedance spectroscopy (EIS) analyzes both the resistive and capacitive properties of a medium or electrodes. Many researches have been conducted with conventional EIS, which has been focused on the changes in electrical impedance of a medium resulting from the bacterial growth [1][2]. The impedimetric biosensor, which was devised to increase the selectivity and the sensitivity by incorporating a biologically functionalized detection layer on the surface of the electrode, usually measures electrode or interface impedance.

A variety of impedimetric biosensors have been constructed to monitor various biological reactions at the surface of electrodes by immobilizing biomolecules such as enzymes, antibodies, nucleic acids, cell, and microorganisms [3]. Even though some impedimetric biosensors have been used to detect various microorganisms including *E. coli* O157:H7 [4] and *Salmonella typhimurium* [5], still there is a demand for the performance improvement of the biosensor.

Since characteristics of the electrode, including size, shape, materials, and biological receptors greatly affects the performance of the impedimetric biosensor, modifying the electrode have received attentions. Improvements in the sensitivity of impedimetric biosensor by reducing the size of the interdigitated electrode has been reported [6]. Nanoparticle enhancement may be useful in improving the response of impedimetric biosensors. Nanoparticles have been used as signal enhancement materials in many biosensor applications due to unique electronic, photonic, and catalytic properties with biomaterial. The specific interaction between target molecules and nanoparticle-antibody conjugate induce the change of the electrical properties such as resistance and capacitance, which can be represented as the change of impedance.

The purpose of this paper was to develop a nanoparticle-enhanced impedimetric biosensor for detecting *Salmonella* enteritidis in food samples. To complete the research objective, a modified sandwich assay was devised and performed to evaluate the method.

2. Materials and Methods

2.1. *Bacteria and sample preparation*.

Salmonella enterica serotype *enteritidis (S. enteritidis) ATCC 11076* was used for in the experiments. The bacteria were maintained on brain heart infusion (BHI) agar (1.5%) slants (Difco Laboratories) at 25°C for the duration of this study. Fresh cultures of *S. enteritidis* were prepared by incubating the slant cultures in 5 ml of BHI broth at 37°C with shaking (150 rpm). After 14 h culture cell numbers reached about 1 X 10^9 CFU/ml. In some cases, bacteria were adjusted to approximately the same concentration by using a spectrophotometer (Beckman-Coulter, CA, USA). For sample preparation, selenite broth was purchased from Sigma-Aldrich (MO, USA). Packages of milk were purchased from a local grocery store.

Cell containing buffer was changed to 20 mM phosphate-buffered saline (PBS, pH 7.2) by following procedure. 1 ml of the enriched cell suspensions were centrifuged for 10 min at 5000g and

supernatant was discarded. Collected cell pellet was resuspended in 1 ml of PBS and washed two more times with same procedure. The cells were diluted to appropriate numbers (10^7 – 10^2 cfu/ml) with PBS, and used for the immunoassay. Milk samples were prepared by inoculating 100 µl of the cell suspension into the milk. The cells were also diluted to appropriate numbers (10^7 – 10^2 cfu/ml) with milk. For negative control, PBS or milk, which does not contain cells, was used. Enumeration of the enriched *S. enteritidis* was performed using the standard plate count (SPC) method.

2.2. *Regeants and antibodies.*
Biotinylated rabbit anti-*Salmonella* polyclonal antibody was purchased from ViroStat Inc. (Portland, ME, USA). Bovine serum albumin (BSA), and phosphate buffer were purchased from Sigma (St. Louis, MO, USA). Neutravidin was purchased from Pierce (Rockford, IL, USA). As nanoparticles, streptavidin coated quantum dot was purchased from Evident Technologies (New York, USA).

2.3. *Impedimetric biosensor preparation and instrument setup*.
Gold coated interdigitated microelectrodes (IME) were obtained from BAS Inc. (Tokyo, Japan). The gold electrodes were created by lithography on the glass substrate. The IME had 65 electrode pairs and 2 mm of the length of the finger electrode. Width and space of the electrode were 10 µm and 5 µm, respectively. The IME were prepared to have active binding sites for *S. enteritidis* as follows. To immobilize capture antibodies on the surface of the IME, 100 µl of 100 µg/ml neutravidin was applied and incubated 20 min at room temperature. The IME were rinsed with PBS-Triton (0.02 M phosphate buffered saline (PBS) containing 0.05 % Triton X-100) and incubated with 100 µl of 20 µg/ml biotinylated anti-*Salmonella* antibody in PBS at room temperature for 10 min. Finally, the IME were rinsed with PBS-Triton again and incubated with 100 µl of 1 mg/ml bovine serum albumen (BSA) at room temperature for 3 min to block non-specific binding sites. After immobilization of capture antibody onto the IME, the impedimetric biosensor was connected to impedance measurement set up. Impedance measurements were performed using a Bode-100 impedance analyzer module (Omicron Electronics GmbH, Klaus, Austria). The set up of the impedance measurement with the impedimetric biosensor is shown in Figure 1.

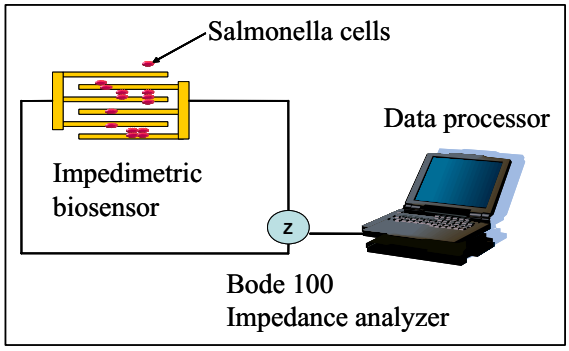

Figure 1. Instrument setup for impedance measurement.

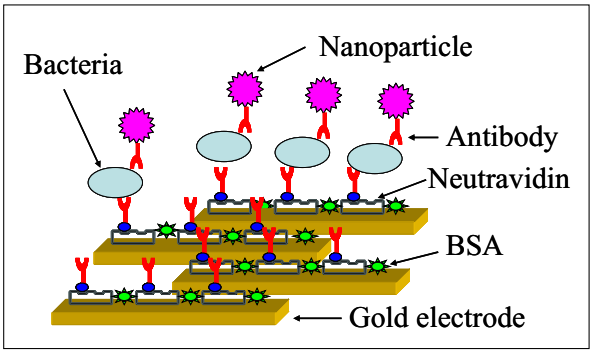

Figure 2. Detection and nanoparticle enhancement principle of the impedimetric biosensor.

A background signal was measured first using PBS as a sample. With the same biosensor, consecutive measurements were performed using serially diluted bacteria samples (10^3 ~ 10^7 CFU/mL) in PBS or milk. To allow the antibody-antigen reaction, the biosensor was incubated with the sample for 3 min before the signal measurement. Between the measurements, the biosensor was rinsed three times with PBS. For additional nanoparticle enhancement experiment, 20 µg/mL of streptavidin conjugated quantum dots were incubated with 40 µg/mL of anti-*Salmonella* polyclonal antibody prior to introduce onto the biosensor. Any unconjugated antibody remaining in the mixture

was removed by column chromatography. The antibody-conjugated nanoparticles were applied onto the biosensor immediately after each sample measurement (Figure 2).

3. Results and Discussion

Optimum input frequency was determined by analyzing frequency characteristics of the biosensor over ranges of applied frequencies from 100 Hz to 400 Hz. At 100 Hz of input frequency, the biosensor was most sensitive to the changes of the bacteria concentration and this frequency was used for the detection experiments (Table 1).

Table 1. Response of the biosensor over ranges of applied frequencies for 10^6 CFU/mL of *S. enteritidis* in PBS.

Frequency (Hz)	10	100	200	400
Impedance increase from baseline (Ω)	-79	114	26	-24

3.1. *Detection of S. enteritidis in PBS and milk samples*

The responses of biosensors to increasing concentrations of *S. enteritidis* spiked into PBS are shown in Figure 3. Higher concentration of cells in the sample generally increased responses. Because the cells attached onto the surface of the biosensor act as resistors, the larger the number of attached cells increased the impedance. The impedimetric biosensor could detect 10^6 CFU/mL of *S. enteritidis* in PBS. Figure 4 shows the biosensor responses for milk samples. The background signal of milk was higher than that of PBS, and it took more time to reach steady state. After background signal reading was done with plain milk, measurement for milk samples were continued. The impedimetric biosensor could detect *S. enteritidis* in milk but it requires more cells to give positive detection results. Detection limit of the biosensor for *S. enteritidis* in milk was 10^7 cfu/ml. A possible reason of this sensitivity decreases in milk comes from interference of antigen-antibody bindings from complex entity of milk. The milk ingredients could interfere with the signal measurements. Furthermore, the detection could be affected by stressful environment of food which might alter physiology and metabolism of the microorganisms thus interfering antibody binding to bacteria.

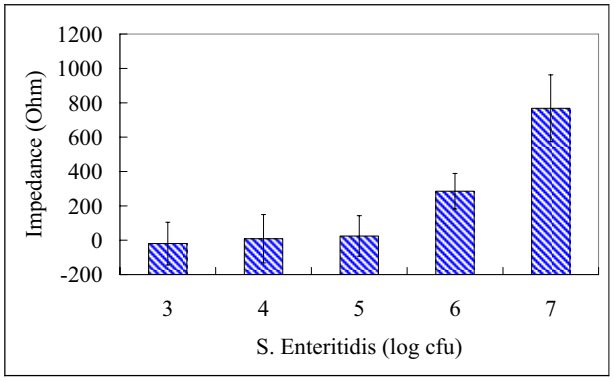

Figure 3. Response of biosensor for different concentrations of *S. enteritidis* in PBS. Detection limit of the impedimetric biosensor was 10^6 CFU/mL.

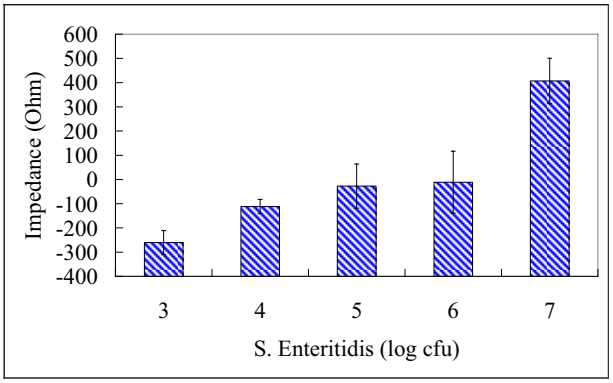

Figure 4. Response of biosensor for different concentrations of *S. enteritidis* in Milk. Lowest detectable cell numbers were 10^7 CFU/mL.

3.2. *Effects of nanoparticles to enhance sensitivity on impedance measurement*

To increase the sensitivity of the biosensor, antibody conjugated nanoparticles were introduced onto the biosensor surface after sample measurement was done. Figure 5 shows responses of *S. enteritidis* spiked PBS samples with antibody-nanoparticle conjugation enhancement for 3 min at room

temperature. With nanoparticle ehancement, the biosensor could detect 10^4 CFU/mL of *S. enteritidis* in PBS. This method also worked for detecting *S. enteritidis* in the milk sample. Figure 6 shows detection of 10^5 CFU/mL of *S. enteritidis* in milk. This sensitivity increase by using nanoparticle as a signal enhancement material can be explained by blocked electron transfer resulted from additional nanoparticle layer formed onto the surface of the electrode. Since the antibody-nanoparticle film is nonconductive, the impedance signal from the biosensor might be amplified.

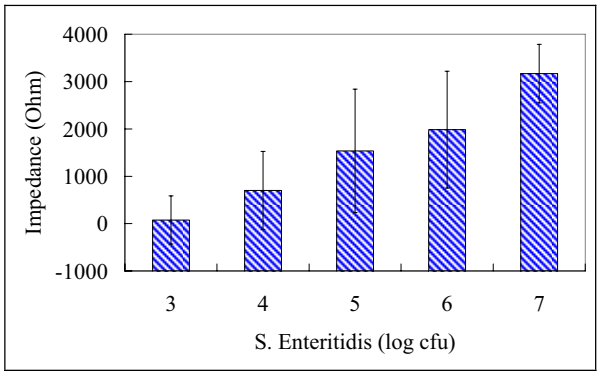

Figure 5. Enhanced response of biosensor for different concentrations of *S. enteritidis* in PBS. Positive result was detected at 10^6 CFU/mL of cell numbers.

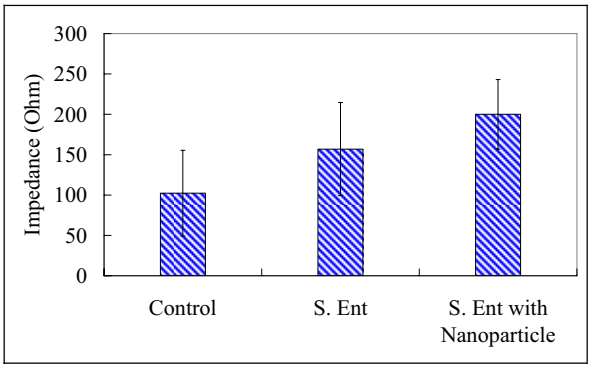

Figure 6. Detection of 10^5 CFU/mL of *S. enteritidis* in milk using nanoparticle enhanced impedimetric biosensor.

By using the nanoparticle as a signal enhancement material, this method could lower the detection limit. For more rapid detection of *S.* enteritidis, the detection method needs further improvement. Possible improvement method includes the use of better antibodies, such as monoclonal antibody, and smaller size of IMEs.

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