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Rapid Detection of *Salmonella enteritidis* in Pork Samples with Impedimetric Biosensor: Effect of Electrode Spacing on Sensitivity

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Abstract Frequent outbreaks of foodborne illness have been increasing the awareness of food safety. Conventional methods for pathogen detection and identification are labor-intensive and take days to complete. Some immunological, rapid assays are developed, but these assays still require prolonged enrichment steps. Recently developed biosensors have shown potential for the rapid detection of foodborne pathogens. In this study, an impedimetric biosensor was developed for rapid detection of *Salmonella entritidis* in food sample. To develop the biosensor, an interdigitated microelectrode (IME) was fabricated by using a semiconductor fabrication process. Anti-*Salmonella* antibodies were immobilized based on neutravidin-biotin binding on the surface of the IME to form an active sensing layer. To evaluate the effect of electrode gap on sensitivity of the sensor, 3 types of sensors with different electrode gap sizes (2, 5, and 10 μm) were fabricated and tested. The impedimetric biosensor could detect 10^3 CFU/mL of *Salmonella* in pork meat extract with an incubation time of 5 min. This method may provide a simple, rapid, and sensitive method to detect foodborne pathogens.

Keywords: biosensor, *Salmonella*, interdigitated microelectrode, impedimetric, immunosensor

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 hr after consuming a contaminated food or beverage. The pathogen is usually associated with raw or undercooked eggs and poultry. *S. enteritidis* outbreaks continue to occur, and *S. enteritidis*-related outbreaks from various food sources have increased public awareness of this pathogen.

Conventional methods for pathogenic microorganism detection and identification involve prolonged multiple enrichment steps. Some rapid immunological assays are available with sensitivity comparable to those of the conventional methods (1,2). One common rapid detection method is based on impedance characteristics of electrodes in a medium where bacteria reside. Impedance measurement methods have analyzed both the resistive and capacitive properties of the medium or electrodes. Most research conducted with conventional impedimetric methods has focused on the changes in electrical impedance of a medium resulting from the bacterial growth (3,4). Impedance changes of the medium result from the release of ionic metabolites from live bacteria. Some researchers measured impedance changes of both the medium and electrodes (5,6). Even though the impedimetric method reduces the time for the detection of bacteria in food, it still requires hours of incubation time to detect low number of bacteria.

Recently, biosensors have shown great potential for rapid detection of foodborne pathogens (7). They are capable of directly monitoring the receptor-analyte 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, which was devised to increase the selectivity by incorporating a biologically functionalized detection layer on the surface of the electrode, usually measures electrode or interface impedance. The impedimetric biosensor enables qualitative and quantitative monitoring of bacteria by measuring the changes in the electrical impedance due to the presence of bound molecules.

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, cells, and microorganisms (8). In fact, some impedimetric biosensors have been used to detect various microorganisms including *E. coli* O157:H7 (9) and *Salmonella typhimurium* (10). However, more research is needed to improve the performance of *Salmonella* detection in food.

In this study, impedimetric biosensors were developed and evaluated for detection of *S. enteritidis* in food samples. To increase sensitivity of the biosensor, different specifications of interdigitated electrodes were fabricated and tested. The impedimetric biosensors were evaluated by performing *S. enteritidis* detection in phosphate buffered saline (PBS) and food samples.

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Materials and Methods

Materials Most reagents including bovine serum albumin (BSA), phosphate buffered saline (PBS), Triton-X 100, acetone, NaOH, and ethyl alcohol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Neutravidin was purchased from Pierce (Rockford, IL, USA). Biotinylated rabbit anti-*Salmonella* polyclonal antibody was purchased from ViroStat Inc. (Portland, ME, USA). For sample preparation, selenite broth was purchased from Sigma-Aldrich. *Salmonella enterica* serotype *enteritidis* was obtained from Dr. Bhunia's laboratory (Purdue University, IN, USA) and used for the experiments. The bacteria were maintained on brain heart infusion (BHI) agar (1.5%) slants (Difco Laboratories, Detroit, MA, USA) at 25°C for the duration of this study.

Interdigitated microelectrode (IME) sensor fabrication The IME sensor was fabricated by using a photolithographic processing method. Photoresist was spun onto a glass wafer and patterned using a mask. Then a 50 nm Cr layer adhesive layer and 100-nm thick interdigitated gold electrode was deposited over the Cr layer by sputtering. Active sensing area was created by soaking the wafer in the acetone to lift off the metal not adhering to the grass substrate. Three different sensor types with different electrode gap sizes (2, 5, and 10 μm) were fabricated to evaluate the effect of electrode specification on the sensitivity of the sensor. Each sensor had a 3 mm^2 active sensing area. Each electrode finger had a length of 990 μm and a fixed width of 10 μm. Each sensor had a different number of electrodes according to electrode spacing. Total numbers of electrodes were 250, 200, and 150 for 2, 5, and 10 μm of gap size, respectively. The IME sensor design is shown in Fig. 1. The sensor surface outside of the electrode finger region was coated with insulation epoxy to maintain a fixed sensing-area throughout experiments.

Impedimetric biosensor preparation The impedimetric biosensor was fabricated by immobilizing biological binding ligands on the IME sensor. Before the immobilization process, the IME sensor surface was cleaned with acetone, a solution of 0.1 M NaOH and 1% Triton X-100, 100% ethyl alcohol, and deionized water in sequence. The biosensor was prepared to have active binding sites for *S. enteritidis* as follows. Firstly, 1 mg/mL biotinylated BSA (bBSA) was applied and incubated overnight at room temperature to form a linking protein film on the sensor surface. Secondly, 0.1 mg/mL neutravidin was applied and

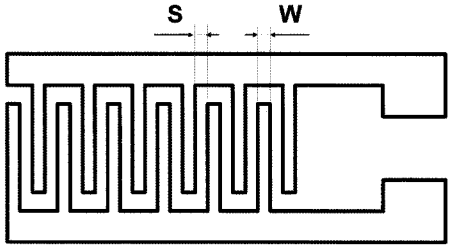


Fig. 1. IME sensor design that has a large number of interdigitated electrode pairs. S and W denote electrode gap size and width, respectively.

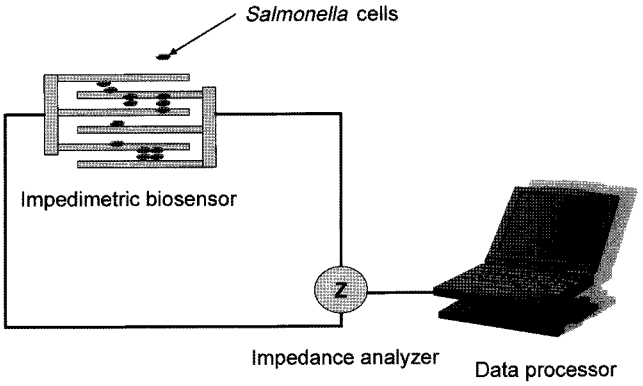


Fig. 2. Impedimetric biosensor signal measurement set up.

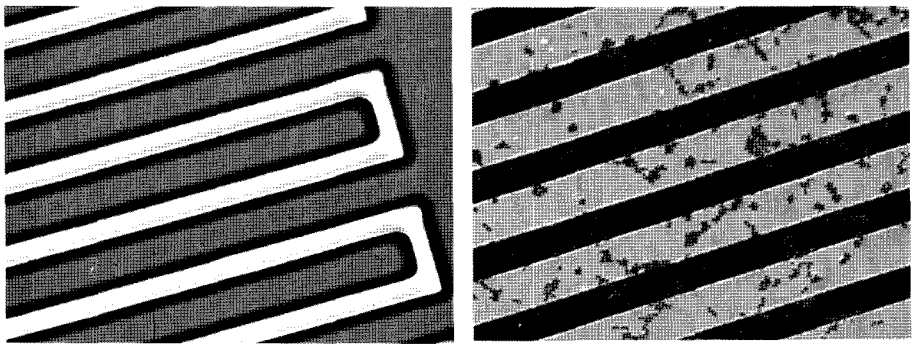
captured onto the physisorbed film of bBSA by incubating 20 min at room temperature. Thirdly, the IME was incubated with 100 μL of 50 μg/mL biotinylated anti-*Salmonella* antibody in PBS (pH 7.2) at room temperature for 10 min. Finally, the IME was incubated with 100 μL of 1 mg/mL BSA at room temperature for 5 min to block non-specific binding sites. Between each step, the IME was rinsed with PBS to remove unbound reagents.

Instrument setup After immobilization of capture antibody onto the IME, the impedimetric biosensor was connected to an impedance measurement set up. Impedance measurements were performed using an HP 4194A impedance analyzer (Agilent Technologies, Palo Alto, CA, USA). The impedance magnitude of the biosensor was measured at frequencies ranging between 100 Hz to 1 MHz with a 50 mV (amplitude) excitation voltage. To measure only the impedance of the biosensor, impedance calibration for the wiring and probes was done before the measurements. Measured impedance signal was sent to the data processor via a general purpose interface bus (GPIB) connection. The set up of the impedance measurement with the impedimetric biosensor is shown in Fig. 2.

Sample preparation 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 hr culture cell numbers reached about 1×10^9 CFU/mL. In some cases, bacteria were adjusted to approximately the same concentration by measuring optical density using a spectrophotometer (Beckman-Coulter, Fullerton, CA, USA).

Cell containing buffer was changed to 20 mM PBS by the following procedure: 1 mL of the enriched cell suspensions were centrifuged for 10 min at 5,000×g and the supernatant was discarded. The collected cell pellet was resuspended in 1 mL of PBS and washed 2 more times with the same procedure. The cells were diluted to appropriate numbers (10^9-10 CFU/mL) with PBS, and used for the experiment.

Food samples were prepared by inoculating 100 μL of the cell suspension into the food extracts. The cells were also diluted to appropriate numbers (1.1×10^8-1.1×10^3 CFU/mL) with food extract. For negative control, PBS or plain food extract, which did not contain *Salmonella* cells, was used. Enumeration of the enriched *S. enteritidis* was performed using the standard plate count (SPC) method.



(A) (B)

Fig. 3. SEM images of impedimetric biosensors. (A) Clean surface of a biosensor (1,000×), (B) bacteria bound to antibodies immobilized to biosensor surface (1,100×).

For the food sample preparation, packages of pork were purchased from a local grocery store.

Detection of *S. enteritidis* When the impedimetric biosensor and the sample sets were ready, a background signal was measured first using PBS as a sample. With the same biosensor, consecutive measurements were performed using serially diluted bacteria samples in PBS or food extract. To allow the antibody-antigen reaction, the biosensor was incubated with the sample for 5 min before the signal measurement. Between measurements, the biosensor was rinsed 3 times with PBS-Triton (0.02 M PBS containing 0.05% Triton X-100) followed by PBS.

For each sample set, 3 replicate measurements were performed for statistical analysis. At the end of each replicate, the measurement surface of the biosensor was regenerated with a solution of 100 mM glycine adjusted to pH 2.5 by HCl. The limit of detection was calculated as 3 times the standard deviation of the 3 control signals. A change in signal above the control signal for all samples tested was considered a positive result if the change was higher than the limit of detection.

For each experiment, the standard deviation of the mean (SEM) signals from 3 replications with same sample sets was calculated. The error bars on each graph designate ±SEM.

Results and Discussion

Verification of the impedance biosensor *S. enteritidis* was detected by reading impedance changes caused by the attachment of the cells to the anti-*Salmonella* antibodies immobilized on interdigitated gold electrodes. The antibodies were immobilized on the electrode surface by using neutravidin-biotin binding. The impedance across the interdigitated electrodes was measured after the series of sample introductions. Bacteria cells captured by the antibody changed the impedance between the electrodes.

Specification of the biosensor and binding of *S. enteritidis* to antibodies immobilized on the biosensor surface was examined with a scanning electron microscopy (SEM). Figure 3 shows the SEM image (1,000×) of the biosensor before (A) and after (B) the detection assay. Larger magnification (1,100×) of Fig. 3B clearly shows that bacteria were attached to the used biosensor surface after rinsing with PBS-Triton and PBS.

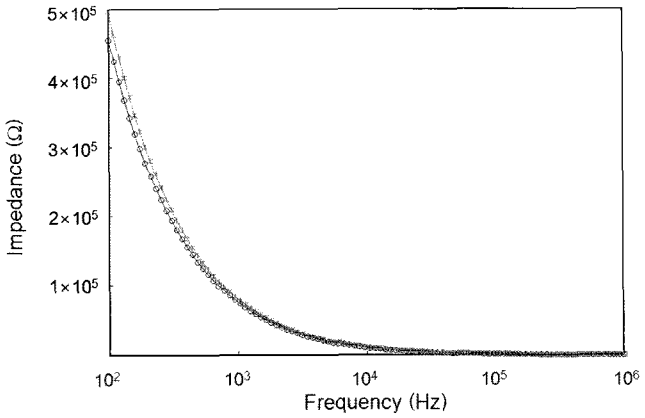


Fig. 4. An impedance spectrum of samples of pure PBS (O) and 10^9 CFU/mL (*).

Figure 4 shows the magnitude of impedance of the biosensor for control and 10^9 CFU/mL of *Salmonella*. A sample containing higher concentration of cells (10^9 CFU/mL) produced higher impedance signal for all the frequency ranges. The signal difference between 2 samples is most notable at low frequency range.

Equivalent circuit model Behavior of real impedance electrodes can be represented by an equivalent circuit model. Equivalent circuit models of electrodes are well documented in the literatures (6,11,12). A simple circuit model of an impedimetric biosensor in contact aqueous solutions is shown in Fig. 5, where C_{di} is the dielectric capacitance of the electrolyte, R_s is the medium resistance, and C_{dl} is the interfacial impedance between the electrode and the electrolyte.

According to the types of elements in the equivalent circuit, there are 3 different regions in the impedance spectra. The dielectric region is in high frequency range over 1 MHz. In this region, the dielectric capacitance of the medium (C_{di}) mostly contributes to total impedance. The intermediate range is the resistive region in which conduction of ions (R_s) in the medium dominates the signal. In the low frequency range lower than 100 kHz, double layer capacitance determines the signal. At this region, cell attachment on the biosensor surface greatly influences the signal. To measure

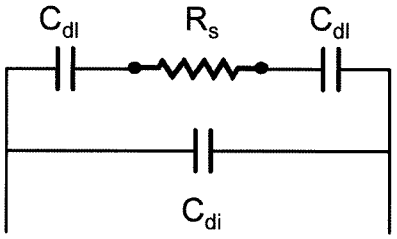


Fig. 5. The equivalent circuit of the impedimetric biosensor. C_{dl} is the double layer capacitance; R_s is the resistance of the medium; C_{di} is the dielectric capacitance of medium.

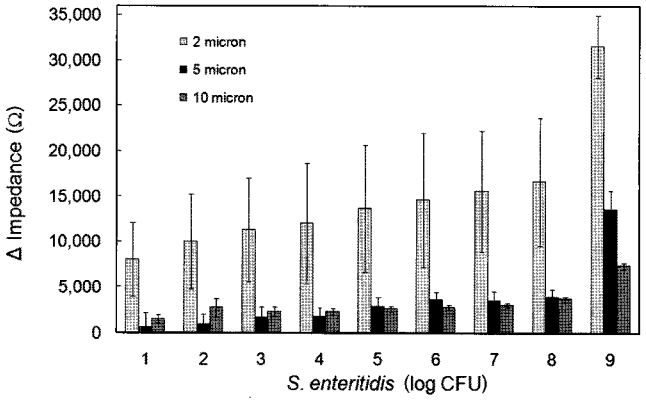


Fig. 6. Impedance changes of biosensors for different concentrations of *S. enteritidis* in PBS (at 100 Hz).

the signal that is most sensitive to the attachment of *S. enteritidis* to the antibodies on the biosensor surface, impedance signals at 100 Hz were used for data analysis.

Detection of *S. enteritidis* The impedance changes of biosensors with increasing concentrations of *S. enteritidis* spiked into PBS are shown in Fig. 6. The impedance change over the background of the biosensor increased with the number of bacteria in the sample. Since, a sample with high cell concentrations tends to have more cells being attached to antibodies immobilized on the sensor surface, higher concentration of cells in the sample generally increased responses. Because the cells attached onto the surface of the biosensor act as resistors, the attached cells resulted in increased impedance.

The impedance changes of 5 and 10 μm gap size biosensors increasing with the cell concentration of the sample. In the Fig. 6, impedance signal of 10 μm gap size biosensor for 10^2 CFU/mL sample is larger than that of 10^3 CFU/mL sample resulted from one abnormal measurement among 3 replicates. Without the abnormal data, the impedance responses of the 10 μm gap size biosensor were proportional to the cell concentration of the sample similar to those of 2 and 5 μm gap size biosensors. For all 3 types of the biosensors, there were significant signal increases at 10^9 CFU/mL of cell numbers. This verifies that the increased signals are from cell attachment on the sensor surface.

Figure 7 shows impedance signals for 10 μm gap size biosensor at 1 MHz for same data set as Fig. 6. Since conduction of ions in the medium dominates the signal at this frequency, signal increase at 10^9 CFU/mL of cell numbers

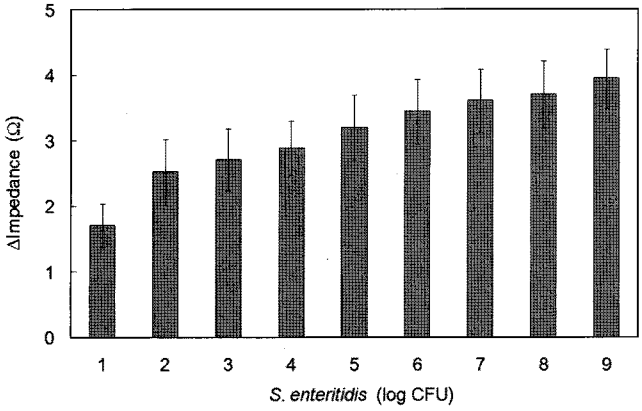


Fig. 7. Impedance change of biosensor for different concentrations of *S. enteritidis* in PBS (10 μm gap size; at 1 MHz).

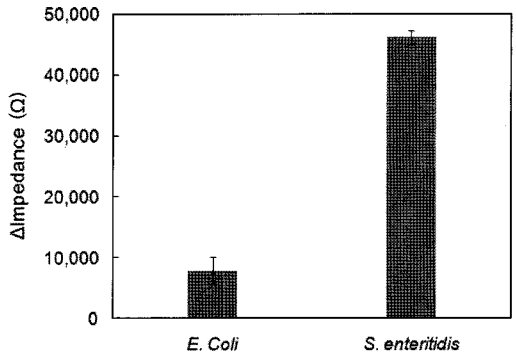


Fig. 8. Specificity of the biosensor for *S. enteritidis* (10^9 CFU/mL) when compared with *E. coli.* (10^9 CFU/mL) (5 μm gap size; at 100 Hz).

is much less than 100 Hz data. Also, the impedance responses are proportional to the cell concentration of the sample.

Specificity and sensitivity When the biosensor was evaluated for its detection with *E. coli* at approximate concentrations of 10^9 CFU/mL each, *S. enteritidis* mixed with *E. coli* showed a stronger signal (46,234 kΩ) than *E. coli* alone (7,895 kΩ) (Fig. 8). These suggest that a specific signal for *S. enteritidis* could be acquired even in the presence of common food contaminants.

Figure 9 shows impedance changes of the 2, 5, and 10 μm gap size biosensors for pork samples. The impedance signal of control sample, which is plain food matrix with no bacteria, was higher than that of PBS due to food particles, or other microorganisms in the sample. After impedance signal for control sample was measured, measurements for pork samples with inoculated *S. enteritidis* cells were continued.

Impedance change of biosensors for the spiked food samples also shows proportional relationship to the cell concentration of the sample. Linear relationships between the impedance signal (Im) and the cell concentration (log N) were found. The regression equations are Im=1,257 log N+9,394 with R^2=0.92, Im=7,041 log N+27,375 with R^2=0.99, and Im=1,536 log N+33,279 with R^2=0.86 for 2, 5, and 10 μm gap sensors, respectively. Detection limits of

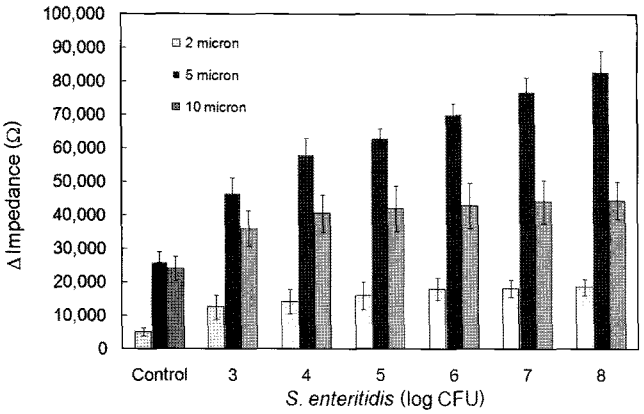


Fig. 9. Impedance changes of biosensors for different concentrations of *S. enteritidis* in pork samples (at 100 Hz).

the biosensors are shown in Table 1. Both the 2 and 5 μm gap sensors could detect 10^3 CFU/mL concentrations of *S. enteritidis* in pork samples. However, the 10 μm gap sensor could detect only 10^7 CFU/mL concentrations of *S. enteritidis* in pork samples.

Effect of the electrode spacing on sensitivity The better detection limit of the 2 and 5 μm gap sensors may be explained by the dimensions of the micro-sized interdigitated electrodes similar to *Salmonella* cell sizes. The performance of the impedimetric biosensor could be affected by many factors including electrode characteristics, biological receptors, and sample matrix. Since, electrode characteristics including size, shape, and materials are relatively easy to modify and to estimate the behavior, modification of electrodes has received attention. Recent popularization of microelectro-mechanical systems (MEMS) technologies enables fabrication of micro-sized electrodes, which are about same or slightly larger size than bacteria size. By using a microelectrode, larger area of the sensor surface could be covered by bacteria. Improvements in the sensitivity of the impedimetric biosensor by using a micro-size IME electrode has been reported (9,12).

The majority of the published papers on the detection of bacteria using IME impedance sensors have focused on the electrical characteristic changes of the culture medium. The detection time of the measured impedance changes in the culture medium method tends to depend on the initial number of bacterial cells present in the sample. If the number of cells present in the sample is very small, the detection time can take relatively long because of the time required for detectable medium resistance changes occurred from cell metabolism. It was reported that less than 10 CFU/mL bacteria in the sample required over 9 hr (6). The detection time for 10^3 CFU/mL initial cell number was reported as 4.6 hr by same authors.

The impedimetric biosensor developed in this research could detect 10^3 CFU/mL concentrations of *S. enteritidis* in pork samples in less than 10 min. Furthermore, the impedimetric biosensor maximizes the impedance change at the surface of the interdigitated electrodes. Utilizing the impedance signal near the vicinity of the electrode surface may reduce the interference of food particulates in bulk solutions.

In this research, impedimetric biosensors for detecting *S. enteritidis* in food samples were developed using an interdigitated microelectrode-based immunosensor. The impedance magnitude of the biosensor was measured at frequency ranges between 100 Hz to 1 MHz with a 50 mV (amplitude) voltage excitation. At 100 Hz, the biosensor was most sensitive to the attachment of the bacteria on the biosensor surface and this frequency was used for analysis. The sensors were evaluated to detect *S. enteritidis* in PBS and pork samples. The impedance signal change over the background of the biosensors was proportional to the number of bacteria in the PBS. The 2 and 5 μm gap sensors could detect 10^3 CFU/mL of *S. enteritidis* in pork samples.

Acknowledgments

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Table 1. Detection limits of biosensors with different gap size in pork samples

Gap size (μm)	Detection limit (kΩ)	Lowest detectable cell numbers (CFU/mL)	Response at the lowest detectable cell numbers (kΩ)
2	11.3	10^3	12.6
5	42.8	10^3	46.3
10	43.5	10^7	44.2

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