Rapid and sensitive biosensor for *Salmonella*

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Abstract

The rapid and sensitive detection of *Salmonella typhimurium* based on the use of a polyclonal antibody immobilized by the Langmuir–Blodgett method on the surface of a quartz crystal acoustic wave device was demonstrated. The binding of bacteria to the surface changed the crystal resonance parameters; these were quantified by the output voltage of the sensor instrumentation. The sensor had a lower detection limit of a few hundred cells/ml, and a response time of < 100 s over the range of 10^2 – 10^{10} cells/ml. The sensor response was linear between bacterial concentrations of 10^2 – 10^7 cells/ml, with a sensitivity of 18 mV/decade. The binding of bacteria was specific with two binding sites needed to bind a single cell. The sensors preserve ~75% of their sensitivity over a period of 32 days. © 2000 Elsevier Science S.A. All rights reserved.

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1. Introduction

Every year, up to 33 million Americans become ill due to foodborne pathogens, representing an estimated \$14.2 billion in lost productivity (Buzby et al., 1996). Pathogenic bacteria cause over 90% of foodborne illness. Current industrial methods of detecting pathogenic bacteria require a minimum of 6–48 h (Rodrigues and Kroll, 1990), by which time portions of the food may have been distributed, marketed, sold, and eaten before a problem is even detected. Recently, antibodies immobilized by covalent coupling and self-assembly onto acoustic wave devices were utilized to construct bacterial sensors capable of detecting specific bacteria within 25–90 min with a lower detection limit of 10^5 cells/ml (Ye et al., 1997; Park and Kim, 1998; Pyun et al., 1998). A comparative study of several antibody immobilization techniques for preparing immunosensors including covalent binding, physical adsorption, and the Langmuir–Blodgett (LB) technique, concluded

that the conventional LB technique produced the highest density of coating, but with very high non-specific binding and poor reproducibility, indicating that the film did not have a well organized structure (Ahluwalia et al., 1992). This research was conducted to improve the speed, sensitivity, and stability of antibody-coated biosensor for the detection and quantification of *Salmonella typhimurium*. Specially prepared monolayers of antibodies were immobilized by the LB technique on the surface of an acoustic wave device. The results presented here show that a rapid and sensitive *Salmonella* biosensor can be developed using this method.

2. Experimental

2.1. Cultures

S. typhimurium and *Escherichia coli* O157:H7 cultures from the Auburn University culture collection were used in the experiments. Each culture was confirmed for identity using traditional biochemical, cell morphol-

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ogy, and serologic tests. The cultures were maintained on Trypticase agar (TSA) slants.

2.2. Growth of cultures and dilutions

Each culture was streaked for isolation on TSA plates before inoculation of a fresh culture to trypticase soy broth for overnight incubation at 37°C in a shaking water bath incubator. Then, the cells were washed by performing centrifugation (3500 rpm for 10 min) of the broth and resuspending the cells in 10 ml sterile phosphate buffered saline (PBS) (pH 7.0) before repeating the centrifugation step and resuspending in 2 ml PBS. Aseptic procedures were used throughout the steps taken to produce the test volumes of bacteria. Serial dilutions were made with PBS. All tubes were shaken before each pipetting to assure mixing before delivery.

2.3. Colony forming unit (cfu) determinations

The number of viable cells in each dilution was determined by spread plating 0.1 ml of each dilution onto duplicate plates of TSA, and incubating 48 h before making a final count of the cfu's and calculating the average cfu based on dilutions yielding 30–300 colonies/plate. The tubes with diluted cells were immediately placed on ice and delivered to another laboratory for testing with the sensor.

2.4. Antibodies

Antibodies used as capture antibodies on the membranes attached to the sensor were obtained from Oxoid, Inc. (Ogdensburg, NY). For *S. typhimurium*, a polyvalent somatic O antibody specific for most *Salmonella* serovars was employed. Reactivity was checked against the target bacterium by performing a slide agglutination test in which the cells agglutinated in the presence of the specific antibody.

2.5. Procedures for examining the reactivity of antibodies

Three types of tests were employed to examine the reactivity of antibodies: (1) a dot blot ELISA test using nitrocellulose or nylon filters with antigen fixed and subjected to chromogenic anti-mouse (for monoclonal antibody) or anti-animal-based antibody conjugated with enzyme assay; (2) kit tests for target organisms; and (3) agglutination tests.

2.6. Monolayer techniques

2.6.1. Surface film balance

Measurements of surface pressure were performed using a Langmuir–Blodgett film balance KSV 2200 LB

(KSV-Chemicals, Finland). This fully computerized system contains a Wilhelmy-type surface balance (range 0–100 mN/m; sensitivity 0.05 mN/m), a Teflon trough (45 × 15 cm²), a variable speed motor-driven Teflon barrier (0–200 mm/min), and a laminar flow hood. The trough was mounted on a 200 kg marble table. Vibration control was provided by interposing rubber shock absorbers, and by mounting the laminar flow hood on a separate bench. Surface pressure was monitored by use of a sandblasted platinum plate of 4 cm perimeter. Temperature of the subphase was controlled ($\pm 0.1^\circ\text{C}$) by water circulation through a quartz tube coil on the bottom of the trough. Temperature was measured by a thermistor located just below the water interface. Surface pressure data are collected during slow, steady-state compression of the monolayers. The monolayer elasticity $E = -A(\partial P/\partial A)_T$ as a function of the surface pressure, was calculated directly from the pressure–area isotherms (Vodyanoy et al., 1990).

2.6.2. Monolayer formation and deposition

2.6.2.1. Phospholipid monolayers. Phospholipid solutions were spread on the surface balance as hexane solutions (1 mg/ml) containing 2% ethanol (Ito et al., 1989). The subphase used in the experiments was a solution containing 55 mM KCl, 4 mM NaCl, 0.1 mM CaCl₂, 1 mM MgCl₂ and 2 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS) made with deionized double distilled water (pH adjusted to 7.4 with KOH).

2.6.2.2. Monolayers with immobilized antibodies. The quartz crystals with gold electrodes for the acoustic wave device were cleaned by treatment with 50% (v/v) HNO₃ and were rinsed in running distilled water until the acid was completely removed. The quartz crystals were then dried and stored until further use. The monolayer was formed on the air–liquid interface by allowing the spreading solution to run down an inclined wettable planar surface that is partially submersed into the subphase. A total of 150 μl of the antiserum was spread on the subphase surface by allowing it to flow down a wet glass plate that crossed the interface. The flow rate down the plate was maintained at about 0.1 ml/min. After spreading the glass plate was removed, and the monolayer was allowed to equilibrate and stabilize for 10 min at $19 \pm 0.1^\circ\text{C}$. The monolayer was then compressed at a rate of 30 mm/min and the vertical film deposition was carried out with a vertical rate of 4.5 mm/min and at a constant surface pressure of 23 mN/m. Seven monolayers of the *Salmonella* antibody film were transferred to the gold surface of the quartz crystals in this manner. Monolayers containing antibodies were transferred at a constant surface pres-

sure onto the round ($d = 1''$) quartz crystals with gold electrodes for acoustic wave device measurements, or onto standard microscope slides for visual observations and cell counting.

2.7. Bacteria binding measurements

2.7.1. Acoustic wave device measurements (AWD)

Measurements were carried out using a PM-700 Maxtek plating monitor with a frequency resolution of 0.5 Hz at 5 MHz. The device was capable of working in both single and dual probe modes. Voltage output of the Maxtek device was recorded and analyzed using a standard personal computer, data acquisition card and software. The voltage output from the Maxtek device is directly related to the resonance frequency of the quartz crystal sensor. Changes in the resonance frequency of the quartz crystal sensor were used to monitor the binding of bacteria to the sensor surface. The observed changes in the resonance frequency of the quartz crystal sensor during binding of bacteria is hypothesized to be due both to viscoelastic changes of the LB film-bacteria-near surface fluid media and the mass change associated with the binding of bacteria.

2.7.2. AWD quartz crystal sensors

AT-cut planar quartz crystals with a 5 MHz nominal oscillating frequency were purchased from Maxtek, Inc. Circular gold electrodes were deposited on both sides of the crystal for the electrical connection to the oscillatory circuit.

2.7.3. Binding measurements

The sensor covered with the antibody film was positioned in the probe arm of the instrument just before delivery of test solutions. Immediately after the recording was started, 1000 μ l of the control solution was delivered with a pipette to the dry sensor surface and the voltage was recorded for 4–8 min. Then the test solution was carefully removed with a plastic pipette tip. After removal of the control solution a new recording was initiated and 1000 μ l of solution containing bacteria was added and the same measuring procedure was followed. Temperature of the test solutions was controlled ($\pm 0.1^\circ\text{C}$). In order to examine the specificity of bacteria binding by antibody immobilized in the sensor membrane, the sensor was exposed by the bacteria incubated with the solution containing free antibody for the optimized duration. After all the solutions were tested, the sensor crystal was carefully removed and placed in absolute ethanol until it was cleaned in nitric acid. All the equipment used in the experiment was sterilized in ethanol. The data collected were stored and analyzed off line.

2.8. Dark-field microscopy

Optical observation of bacterial binding was performed with an Olympus microscope fitted with a 100-W mercury lamp illumination source, a polarizer, a Naessens dark-field condenser (COSE Corp., Canada) and a $100\times$ objective (oil, NA 1.4). The dark-field images (Vodyanoy et al., 1994) were directed to a DEI-470T Optronics CCD Video Camera System (Optronics Engineering, CA). The system provided real-time, direct-view optical images of high resolution. The samples needed no freezing, dehydration, staining, shadowing, marking, or any other manipulation. They were observed in the natural aqueous environment. A direct count of bacteria was used to determine their concentrations in liquid samples. The count of bound bacteria in conditions of large general concentrations of motile cells was used to estimate the surface concentration of specific antibody.

2.9. Binding equations

The purpose of this section was to construct a quantitative description of binding by using known binding equations, and to describe the interaction of antigens and antibodies and resulting complexes in terms of binding parameters. The applicability of the binding equations to the antigen–antibody system is not trivial and special considerations were required. The reaction between antigen molecules and an antibody can be schematically presented as:

where n is the number of molecules of antigen bound to a single antibody.

The association binding constant (K_b) for this reaction can be defined as (Kuchel and Ralston, 1988):

$$K_b = [\text{AbAg}_n]/([\text{Ab}][\text{Ag}]^n) \quad (2)$$

If we ignore the number of antibodies bound to nonspecific molecules, then the total number of antibodies (C_{Ab}) is composed of free antibodies and antibodies bound to antigen molecules:

$$C_{\text{Ab}} = [\text{Ab}] + [\text{AbAg}_n] \quad (3)$$

Combining Eqs. (2) and (3), we can determine the fraction of antibodies occupied by antigens:

$$Y = [\text{AbAg}_n]/C_{\text{Ab}} = K_b[\text{Ag}]^n/(1 + K_b[\text{Ag}]^n) \quad (4)$$

The ratio of occupied and free antibodies can be defined as

$$Y/(1 - Y) = K_b[\text{Ag}]^n \quad (5)$$

Taking the logarithm of both sides, we get

$$\log(Y/(1 - Y)) = \log K_b + n \log[\text{Ag}] \quad (6)$$

A plot of the left-hand side of Eq. (6) versus $\log[\text{Ag}]$ is known as a Hill plot. It gives an estimate of n from the slope, K_b from the ordinate intercept, and EC_{50} at the point when $Y = 1 - Y$. Non-specific binding can be estimated from the saturation curve for antigen binding to a homogeneous antibody population. The total binding includes a component of non-specific binding, which is non-saturable; the remainder is specific binding which saturates at high concentrations of antigens.

2.10. Longevity experiments

Thirty-two *Salmonella* antibody sensors were fabricated by transferring seven monolayers of *Salmonella* antibody serum to each quartz crystal substrate LB technique. Half of the crystals was stored in the refrigerator at 5°C and the other half was stored in a dust free container at 25°C. Each set of crystals was divided into groups of four crystals. The control group was tested immediately after production. The three groups from both refrigerated and the room temperature exposed crystals were tested after 16, 32 and 64 days as described below. The antibody coated crystal was positioned in the holder of the acoustic wave device and the output voltage of the balance was recorded for 8 min after 1000 μl of the following solutions were applied in sequence: subphase solution, 1/625, 1/125, 1/25, 1/5 dilutions of a concentrated *Salmonella* suspension and the undiluted bacterial suspension. The bacteria count was measured each day and the concentrations were calculated accordingly. The concentration of bacteria ranged from 10^6 to 10^{10} cells/ml. The dose-response experiments were carried out at a temperature of 25°C.

3. Results and discussion

3.1. Monolayers containing *Salmonella* antibody

Many features of antibody immobilization originate from the very nature of the antibody itself. Typical antibodies are Y-shaped molecules (2 Fab plus Fc

immunoglobulin structure) with two antigen binding sites located on the variable region of the Fab fragments. All classes of antibody produced by B lymphocytes can be made in a membrane-bound form and in a soluble secreted form (Alberts et al., 1994). The two forms differ only in their carboxyl terminals: the membrane-bound form has a hydrophobic tail (Fc) which anchors it in the lipid bilayer of B-cell membrane; whereas, the secreted form has a hydrophilic tail, which allows it to escape from the cell. Only one of these two forms, the form with a hydrophobic tail, is capable of being held by the monolayers. Thus, it is uniquely qualified for use in the Langmuir–Blodgett technique. This form is also suitable for proper alignment and orientation in sensor membranes. Antibodies derived from immunized animals in the form of antisera or purified protein preparations may contain different impurities and antibodies in both membrane-bound and soluble form.

Methods for forming LB monolayers using organic solvents as a spreading carrier, may drag these impurities and both forms of antibodies into the monolayer. These methods may produce monolayers with high densities of antibodies but with residuals of organic solvent, impurities, and entrapped hydrophilic antibodies that destabilize the monolayer and modulate antigen–antibody interactions. The monolayer in the present work is formed on the air–liquid interface by allowing the spreading solution to run down an inclined wetted planar surface that is partially submersed into subphase (Fig. 1). Membrane vesicles (natural components of serum, or the artificial lipid vesicles) are positioned on the wet slide at the edge of a positive meniscus of liquid, at the liquid–air interface. The hydrophobic antibodies are bound to the vesicular membrane, while hydrophilic antibodies and some impurities are suspended inside the vesicle. When surface forces rupture the vesicle, it splits into a monolayer, and purification occurs. Membrane-bound antibodies are left bound to the newly created monolayer, but soluble antibodies and impurities go into the subphase beneath the monolayer. When the monolayer is compressed and transferred onto the sensor surface there are only membrane-bound antibodies surrounded by compatible lipids.

The steady-state surface pressure isotherms collected during slow compression of the monolayers with *Salmonella* antibodies were consistent with those obtained with ω -tricosenoic acid monolayers containing IgG F5 antibodies (Barraud et al., 1993). The elasticity as a function of the surface pressure, calculated directly from the isotherms, was characterized by the attainment of a maximum of about 36 mN/m at 10 mN/m of surface pressure (Fig. 2). The maximum elasticity is higher than one found for pure protein monolayers (~ 10 mN/m at $A \approx 1$ m²/mg) (Davies and Rideal,

Fig. 1. Method of monolayer formation from the lipid vesicle containing hydrophilic and hydrophobic antibodies (explanations in text).

Fig. 2. The monolayer elasticity as a function of the surface pressure calculated for monolayer containing antibodies for *Salmonella typhimurium*. The monolayer elasticity $E = -A(\partial P/\partial A)_T$ as a function of the surface pressure, is calculated directly from the pressure–area isotherm. The isotherm was obtained by averaging of three runs. The mean standard deviation is 0.25 mN/m.

Fig. 3. Typical response curves obtained by exposing the sensor to buffer solutions containing different concentrations of the bacteria as indicated in the figure. PBS with no bacteria was used as a reference. Approximately 480 data points taken once a second during 8 min represent each line.

Fig. 4. A typical voltage response to step changes in concentration of *Salmonella typhimurium* is shown in Fig. 3. The initial time of the response ($\tau_1 = 8 \pm 1$ s) does not depend on the bacterial concentration. For each bacterial concentration, the sensor signal approaches a steady-state value, corresponding to that concentration within 100 s ($\tau_2 = 79 \pm 20$ s). The smooth fine is a least square second-degree exponential decay curve.

1963), and it agrees well with those found for cell membrane protein monolayers (Blank et al., 1979). When compared with pure protein films, the higher values of elasticity of lipid–protein monolayers indicate a better stability (Davies and Rideal, 1963).

3.2. Response curves

Fig. 3 shows the sensor response curves obtained by exposing the sensor to buffer solutions containing different concentrations of the bacteria. A typical voltage response to a step change in concentration of bacteria is shown in Fig. 4. The initial time of the response ($\tau_1 = 8 \pm 1$ s) does not depend on the bacterial concentration. For each bacterial concentration, the sensor signal approaches a steady-state value, corresponding to that concentration within 100 s ($\tau_2 = 79 \pm 20$ s). The response curves are distinguished from the previous reports for pathogen detection (Table 1) by the fast reaction, the attainment of a steady-state, and very low non-specific binding. These properties can be attributed to the accuracy of the LB technique, to the purification of antibodies during the process of immobilization, and to the placement of the antibody in a compatible environment. It has been reported that 17–90 min were needed to measure bacteria binding by different acoustic wave biosensors (Ye et al., 1997; Park and Kim, 1998; Pyun et al., 1998). Therefore, the biosensor of this work can be regarded as a much more rapid detector for *Salmonella*.

3.3. Dose response

In Fig. 5, curve 1, the mean values of the steady-state output sensor voltages are plotted as a function of

Fig. 5. Dose response plots. Curve 1 represents the mean values of steady-state output sensor voltages as a function of *Salmonella typhimurium* concentrations from 10^2 to 10^{10} cells/ml. The smooth curve is the sigmoid fit to the experimental data ($\chi^2 = 5.9 \times 10^{-5}$). Line 2 shows the dose responses of the sensor exposed to *S. typhimurium* suspensions incubated with *Salmonella* antibodies prior to the exposure. A total of 1.2×10^9 cells were incubated with *Salmonella* antibodies ($\sim 200 \mu\text{g}$) in 1 ml of PBS for 3 h 40 min. The straight line is the linear least squares fit to the data ($R = -0.76$, slope = -9.0×10^{-5} v/decade). Experimental data points were obtained by averaging about 200 data points of each steady-state level of response curves exemplified by Fig. 3; bars are SD.

Fig. 6. A Hill plot. The ratio of occupied and free antibodies is shown as a function of concentration of *Salmonella typhimurium*. The squares represent experimental data. The line represents the least square fit of Eq. (6) with coefficients: $K_d = 230 \pm 50$ cells/ml; $n = 0.45 \pm 0.02$. ($R = 0.98$, $P < 0.001$).

bacteria concentration from 10^2 to 10^{10} cells/ml. The dose response is linear over five decades of bacterial concentration ($R > 0.98$, $P < 0.001$). The sensor sensitivity, measured as a slope of the linear portion of the dose response, is 18 ± 5 mV per decade of *Salmonella* concentration, based on experiments from 112 sensors. The detection limit estimated from the dose response is 350 ± 150 cells/ml. The interaction of *S. typhimurium* with the antibody is specific because the sensor does not respond to the bacteria preincubated with the antibody (Fig. 5, line 2). The dose response curve is in agreement with the Langmuir adsorption equation with dissociation constant ($K_d = 230 \pm 50$ cells/ml) and the Hill coefficient ($n = 0.45 \pm 0.02$) ($R = 0.98$, $P < 0.001$) estimated from Fig. 6. The value of the Hill coefficient indicates that two binding sites are needed to anchor one bacterial cell to the sensor surface (Kuchel and Ralston, 1988). The biosensor has a detection limit below the infectious dose of *S. typhimurium* (10^5 CFU/ml), assuming that the person does not ingest more than 15 fl oz (430 ml) of contaminated liquid (Bergdoll, 1990). The detection limit and the useful analytic range are much better than those previously reported for this type of biosensor (Table 1).

Fig. 7. Longevity of biosensors. A fraction of original sensitivity of *Salmonella* sensors is shown as a function of the time of storage at 5°C (squares) and at 25°C (circles). The sensitivity is defined as a slope of the linear portion of the dose-response curve similar to curve 1 in Fig. 5. Each point represents the average of results obtained for four different samples. The points represent experimental data; bars are SD; and the lines are the least-squares linear regression fits to these data ($R = -0.97$, slope 1.3%/day at 5°C; $R = -0.90$, slope 1.2%/day at 25°C).

3.4. Longevity of biosensor

Longevity experiments show that the sensors preserve ~ 75% of their sensitivity (slope of V versus log concentration curve) over a period of 32 days, and above ~ 25% after 64 days (Fig. 7). The long-term stability was not reported for bacterial biosensors prepared by covalent binding (Ye et al., 1997; Park and Kim, 1998; Pyun et al., 1998). The stability of biosensors observed at room temperature compares well with that found for a piezoelectric immunosensor for hepatitis viruses (Koenig and Graetzel, 1995).

4. Conclusions

The results of this work demonstrate the feasibility of a biosensor based on LB monolayers of an antibody for the rapid and sensitive detection of *S. typhimurium* in liquid samples. Results indicate detection limits of a few hundred cells in less than 100 s. With its large analytic range, low non-specific binding, and rapid attainment of steady-state output, this biosensor could become a useful analytical tool in a large variety of applications.

Table 1
Comparative properties of biosensors

Characteristic	This work	Ye et al. (1997)	Park and Kim (1998)	Pyun et al. (1998)	Konig and Graetzel (1995)
Antigen	<i>Salmonella</i>	<i>Salmonella</i>	<i>Salmonella</i>	<i>E. coli</i>	Hepatitis virus
Range (cell/ml)	10^2 – 10^{10}	5.3×10^5 – 1.2×10^9	9.9×10^5 – 1.8×10^8	3×10^5 – 6.2×10^7	10^5 – 10^{10} viruses
Detection limit (cell/ml)	350 ± 150	$\sim 10^5$	$\sim 10^5$	$\sim 10^5$	10^5
Time constant (min)	1.2 ± 0.2	25 ^b	30–90 ^b	$\sim 17^b$	30
Apparent K_d ^a (cell/ml)	230 ± 50	ND	ND	3.8×10^6	ND
Longevity (days)	32	ND	ND	ND	28

^a K_d dissociation constant estimated from Hill plot; ND, not determined.
^b Measurement time, equilibrium is not attained.

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