analytical chemistry

TECHNICAL NOTE

pubs.acs.org/ac

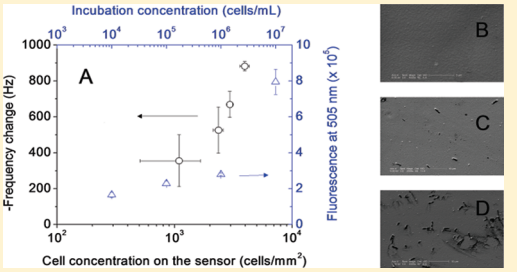
Cell Viability Measurement Using 2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein Acetoxymethyl Ester and a Cantilever Sensor

Sen Xu and Raj Mutharasan*

Department of Chemical and Biological Engineering, Drexel University, Philadelphia, Pennsylvania 19104, United States

Supporting Information

ABSTRACT: Detection of viable pathogenic bacteria has widespread application in food safety and human health. Antibody-based methods require a growth step which limits time-to-results performance. In this study, we use a mass-change sensitive cantilever biosensor and a probe, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM), that accumulates only in live cells inducing a mass-change response to determine the cell viability in a short time. A poly-L-lysine coated sensor immobilized with live *Escherichia coli* JM101 (a surrogate for a pathogenic target) at various concentrations was exposed to BCECF-AM in a flow arrangement. A larger resonant frequency decrease in response to 100 μL of 60 μM BCECF-AM was observed when the sensor surface cell concentration was increased from 1 090 ± 580 to 3 960 ± 370 cells/mm^2 (n = 5). A log−linear relationship between the sensor surface cell concentration and frequency response was obtained in the range of 1 000−4 000 cells/mm^2 and as low as ∼2 000 viable *E. coli* cells were rapidly detected (<1 h).



Rapid detection of live bacterial pathogens is important in monitoring food safety and water quality as well as in medical diagnostics. Selective plating and culturing is the current standard due to its high selectivity and sensitivity. However, the time-to-results (TTR) depends strongly on the growth rate of the pathogen. For the slow growers, confirmation of positive detection could take as long as 16 days.[1] Polymerase chain reaction (PCR) in combination with the plating method can reduce TTR considerably. However, a significant time is still needed to enrich and grow the target microorganism, especially when it is present at low concentrations. PCR techniques without an enriching step do not distinguish between viable and nonviable cells because DNA is a stable molecule and is present in both dead and live cells. Similarly antibody-based biosensors suffer from the deficiency of not distinguishing viable from nonviable cells. Since only the viable cells are virulent, it is important to discern them in a sample. In situations where TTR is long, timely corrective decisions cannot be made. For example, it takes more than 5 h using real time PCR to detect the pathogen *Esherichia coli* O157:H7[2] and more than a day for *Salmonellae*[3] and 2 weeks for *Mycobacterium tuberculosis*.[4] The purpose of this investigation is to examine if a biosensor approach can improve TTR for live cell detection.

The cantilever is an attractive platform for label-free biological detection due to its high sensitivity. Resonant frequency of such a device decreases when the target analyte binds to the surface because of a mass change; for details on cantilever physics, see the reviews.[5,6] Piezoelectric-excited millimeter-sized cantilever (PEMC) sensors have been shown to be very highly sensitive to mass change at femtogram levels.[7] Because of this property, any perturbation to the cell environment that causes it's mass to change in live cells and not in dead cells can be used for detecting live cells. Candidate reagents that fit this requirement are the dyes used for measuring intracellular pH. Acetoxymethyl ester of 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF-AM) is a fluorescent dye that has been extensively used for intracellular pH measurement since 1982.[8] Once it diffuses into the cell, it is hydrolyzed to fluorescent BCECF via the action of intracellular nonspecific esterases known to be present only in live cells and thus can be used as a viability indicator. BCECF carries 4−5 negative charges per molecule at cytosolic pH and thus accumulates within viable cells, and over 80% of the loaded BCECF remains within the cell for at least 2 h.[9] A typical bacterium (∼1 μm) weighs 1 pg, and if the uptake of BCECF-AM is ∼0.1% of its mass, then a single live bacterium mass will increase by 1 fg, which is potentially measurable by a PEMC sensor. Standard sensors such as a quartz crystal microbalance are unable to measure such a small change in mass as its sensitivity is in the range of 10 pg to 1 ng. The dye, BCECF-AM readily diffuses into *E. coli* cells,[10] but its transport rate and the amount of it taken up by a cell has not been reported. In the current study, live *E. coli* cells immobilized on the sensor were exposed to BCECF-AM, and the resulting mass increase was measured via a resonant frequency change. Although detecting viable pathogenic cells is our interest, a nonpathogenic strain *E. coli* JM 101 strain was used as a surrogate in the current study. The approach which combines BCECF accumulation and a highly mass-change sensitive PEMC sensor is shown to be feasible experimentally.

EXPERIMENTAL SECTION

Materials and Reagents. Phosphate buffered saline (PBS, 10 mM, pH 7.4), poly-L-lysine (PLL, 0.1% w/v in H_2O) and AC

Received: October 19, 2010
Accepted: December 2, 2010
Published: January 18, 2011

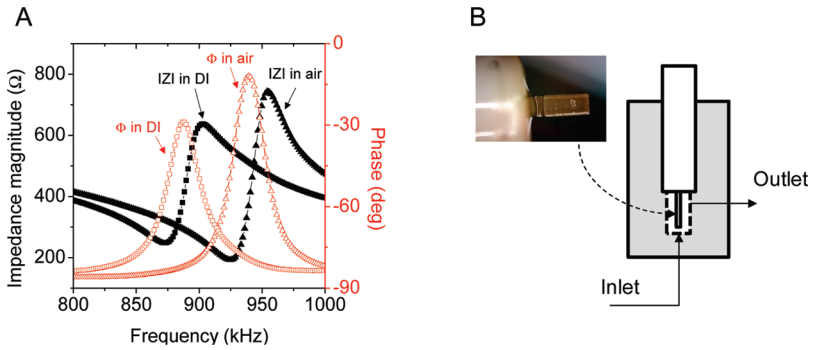


Figure 1. (A) Sensor spectra in air and in liquid. Solid symbols stand for impedance magnitude. Sensor exhibited resonant frequency at 940.71 kHz in air and 873.27 kHz in DI water, which gave a $\Delta f = 67.44$ kHz shift due to the difference in density of the surrounding medium. (B) Flow configuration of the flow cell.

broth were purchased from Sigma-Aldrich (St. Louis, MO). BCECF-AM (B1170) was purchased from Invitrogen (Eugene, OR) and stored at −20 °C. Deionized water (DI, 18 MΩ, Milli-Q system, Millipore, Billerica, MA) was obtained locally. *E. coli* JM101 was cultured in AC broth (prepared as per vendor instructions) at 37 °C overnight. Harvested *E. coli* cells were centrifuged (×6 000 rpm for 3 min) and resuspended in PBS twice. Live cell numbers were counted in a hemocytometer with 0.4% trypan blue. The desired cell concentration samples were prepared by dilution with PBS.

Sensor Preparation. Sensors were fabricated as described previously.[7,11] Briefly, the composite cantilever structure was fabricated with a quartz layer (SPI, West Chester, PA) bonded to a PZT layer (lead zirconate titanate, Piezo Systems, Woburn, MA) by an adhesive. The quartz layer (2.00 mm × 1.00 mm × 0.160 mm) was bonded to the PZT layer (2.70 mm × 1.00 mm × 0.127 mm) ($l \times w \times t$) by a nonconductive adhesive. A layer (10 μm) of Parylene-C was deposited on the sensor surface by chemical vapor deposition in a parylene coating machine (PDS 2010 LABCOTER 2, SCS) to provide insulation for a liquid environment application. A picture of the fabricated sensor is shown in Figure 1B.

Experimental Procedures. Sensor tip (2 mm^2) was freshly sputter-coated with 100 nm gold in a Desk IV sputtering system (Denton Vacuum, Moorestown, NJ). PLL droplets were applied to the gold surface and dried in a chemical hood overnight prior to use.[12] The PLL modified sensor was batch incubated in 1 mL of *E. coli* JM101 cell solution (concentration range from 10^4 to 10^7 cells/mL) for 2 h at 37 °C.

After incubation in a live cell sample, the sensor was rinsed with PBS thoroughly and installed in a homemade flow cell (depicted in Figure 1B). The entire flow apparatus is shown in Figure S2 in the Supporting Information. The flow cell was placed in an incubator, and the temperature was controlled at 30 ± 0.1 °C. A peristaltic pump was used to provide a flow rate of 450 μL/min. The cantilever sensor was connected to an impedance analyzer (HP4192A, Hewlett-Packard), and a LabVIEW program was used for collecting resonant frequency and impedance values in real-time. PBS was used as a running buffer in flow experiments. Upon sensor resonant frequency stabilization in PBS, 100 μL of 60 μM BCECF-AM aqueous solution was injected into the flow loop in recirculation mode. BCECF-AM aqueous solutions were freshly prepared from 10 mM anhydrous DMSO stock solution and diluted to the desired concentration (~1.5 μM final concentration in the flow loop) and used immediately. The volume of the flow loop was ~4 mL, thus it required ~9 min to complete one circulation. The sensor response was assumed to have reached steady state when resonant frequency remained constant for at least 20 min. Subsequently PBS was introduced to rinse the system.

Fluorescence measurement was carried out in a spectrofluorometer (QuantaMaster 40, PTI, Birmingham, NJ). A glass slide cut to fit a cuvette size was incubated in 1 mL of *E. coli* JM101 live cell samples (concentration from 10^4 to 10^7 cells/mL) for 2 h. Then the glass slides were rinsed with PBS and placed in a cuvette filled with 4 mL of PBS; 100 μL of 60 μM BCECF-AM was introduced and incubated for 1 h. After incubation, the glass slide was removed, rinsed with PBS thoroughly, and placed in cuvette for fluorescence measurement. The measurement was done with excitation at 505/439 nm and emission at 535 nm.[13] The ratio of fluorescence of BCECF is a function of pH. The slit widths were 1 nm for excitation and 5 nm for emission. The cuvette was thermostatted at 30 °C for measurement.

SEM Examination of Sensor Surface. Sensors, following the BCECF experiments, were rinsed thoroughly with DI water and air-dried. Subsequently, ~8 nm platinum/palladium (80/20) was deposited on the sensor surface and then examined in a Zeiss Supra 50VP or an FEI XL30 scanning electron microscope. Counting of the cells was typically done within 24 h of the flow experiments.

RESULTS AND DISCUSSION

A group of sensors with identical geometry was selected (m = 30) from a large set that had nearly the same spectral and mass-change sensitivity properties. All sensors exhibited a dominant resonant mode in 940 ± 10 kHz region and showed an air-to-water frequency shift of 70.50 ± 4.50 kHz (m = 30). The small standard deviation of 4.5 kHz suggests a sensitivity variation within 7%. Figure 1A shows the resonance spectra in air and under full immersion in DI water of a typical sensor. PEMC sensors do not suffer from damping in liquids because the Reynolds number (Re) is high (~10^6), and the resonant frequency can be measured with an accuracy of ±20 Hz. This feature enables conducting continuous flow detection experiments in which sample can be changed conveniently and the resulting resonant frequency response is measured. Since the measurement is made *in situ* without removing the sensor from its housing, changes in measured resonant frequency is attributed to the detection response. This measurement format removes the ambiguity that plagues the dip-and-dry method used often with microcantilever sensors.

The number of cells attached to the sensor surface increased with both incubation concentration and incubation time. The

dx.doi.org/10.1021/ac102757q |*Anal. Chem.* 2011, 83, 1480–1483

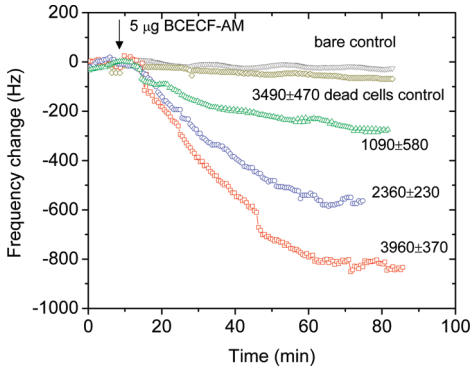


Figure 2. Resonant frequency response to the addition of 100 μL of 60 μM (5 μg) BCECF-AM to sensors with surface cell concentrations (1 090 ± 580, 2 360 ± 230, 3 960 ± 370 cells/mm^2 (n = 5)). Sensor that was immobilized with 3 490 ± 470 cells/mm^2 (n = 3) dead *E. coli* cells gave a very small frequency decrease (62 ± 4 Hz). An additional control (labeled as bare control) with no *E. coli* incubation also yielded no observable response.

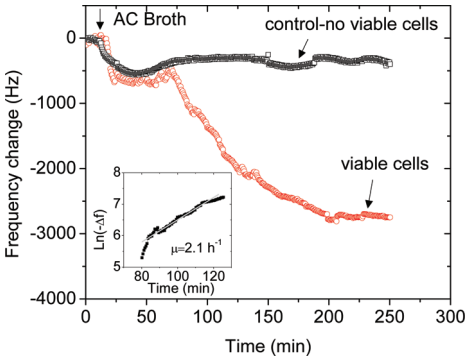


Figure 3. Sensor response upon introduction of AC broth. Sensors at the end of BCECF experiments (Figure 2)s were exposed to growth medium, AC broth. After an initial response due to a density change, a decrease in the resonant freuqency is due to cell growth. The response reaches a constant value, presumably due to stoppage of growth. The inset plot shows a growth response was exponential with a specific growth rate of 2.1 h^{-1}. Control experiments without *E. coli* on the sensor surface gave no response.

relationship between sample concentration and the cell number on the sensor surface bore a log−linear relationship (y = 384.98 ln(x) − 2309.9, R^2 = 0.98) as shown in Figure S1 in the Supporting Information. For the incubation concentrations of 10^4, 10^5, 10^6, and 10^7 cell/mL, the sensor surface cell concentrations were 1 090 ± 580, 2 360 ± 230, 2 950 ± 160, 3 960 ± 370 cells/mm^2 (n = 5), respectively. As a control, sensors coated with gold at the sensor distal tip were exposed to 1 mL of 10^7 cells/mL *E. coli* live cells, and the attached cell was found to be very low (330 ± 100 cells/mm^2, n = 5). In the following part of the discussion, we correlate the sensor response with sensor surface cell concentration as it is the indicator of sensor sensitivity.

In a typical experiment, the sensor was first incubated in 1 mL of live *E. coli* samples (at various concentrations) in batch and then exposed to BCECF-AM in the flow arrangement (Figure S2 in the Supporting Information). The mass of the attached cells increase as BCECF accumulates within and is measured by a resonant frequency decrease. In Figure 2, we show the response of a live *E. coli* JM 101 immobilized sensor to the addition of 100 μL of 60 μM BCECF-AM (5 μg) to the flow loop. In all cases illustrated in Figure 2, the resonant frequency began to decrease ∼5−10 min after the introduction of BCECF-AM. The response took ∼40−50 min to reach a steady state. The time scale of the sensor response is similar to previous reports on the incubation time used (30−60 min).[4,10] As shown in Figure 2, the resonant frequency decrease of 280 ± 10, 563 ± 21, and 864 ± 15 Hz was obtained after BCECF-AM introduction corresponding to the sensor surface cell concentration (measured by SEM) of 1 090 ± 580, 2 360 ± 230, 3 960 ± 370 cells/mm^2 (n = 5), respectively. Impedance response of the sensor corresponding to these three experiments is given in Figure S3 in the Supporting Information. As expected, the response was larger for larger number of cells on the sensor, as total BCECF accumulation is proportional to cell number. Once the sensor response reached steady state, the flow was changed to PBS in a once-through mode. No further change in resonant frequency was observed over the subsequent 20 min period, suggesting BCECF leakage was small or none in that time window. As was shown previously, a PEMC sensor has a sensitivity of ∼1.5 fg/Hz.[14] The uptake and accumulation of BCECF per *E. coli* cell from the three experiments in Figure 2 can be estimated to be 0.17 ± 0.14, 0.16 ± 0.016, 0.15 ± 0.01 fg/cell. On average, the estimated BCECF accumulation in each *E. coli* cell was ∼0.16 fg. Separate control experiments with PLL-prepared sensors without a cell incubation step showed no response to 100 μL of 60 μM BCECF-AM (Figure 2). A second set of controls with killed cells (3 490 ± 470 cells/mm^2, n = 3) immobilized on PLL-prepared sensor yielded a small resonant frequency decrease (62 ± 4 Hz) to the same BCECF-AM addition. The two control experiments indicate that the response observed with live cells to BCECE-AM is due to BCECF accumulation in live cells.

We tested if the cells remained viable subsequent to the BCECF exposure. This was accomplished by introducing growth medium and then examining if the sensor response decreased due to cell growth. The idea is that nutrients in the growth medium would stimulate growth of viable cells on the sensor surface. The growth medium, AC broth, was introduced into the flow loop in recirculation mode after a detection experiment. The temperature of the flow cell was adjusted to 37 °C to facilitate growth. As shown in Figure 3, upon the introduction of 4 mL AC broth, resonant frequency showed a density-induced decrease of ∼600 Hz and reached a new steady state value within 20 min. This response to liquid density change was reported previously.[15,16] The resonant frequency then began to decrease after a lag of ∼50 min, and continued to decrease for a further 140 min, resulting in a total resonant frequency change of ∼2,100 Hz. From the exponential growth phase one can calculate the growth rate (μ) as 2.1 h^{-1}, which was comparable to literature values.[17,18] Control experiments that exposed the sensor with no immobilized cells to AC broth gave no response except the initial density response due to switching from PBS to AC broth. From the cell growth experiments, we confirm that the cells in the BCECF experiments were alive and can be stimulated to grow if nutrients are supplied.

It is of interest to investigate the relationship between the response of the sensor to BCECF-AM and the cell concentration on the sensor surface. As shown in Figure 4A, the resonant frequency decrease in response to 100 μL of 60 μM (5 μg) BCECF-AM correlated with cell concentration on the sensor surface in a log−linear fashion (y = 426.43 ln(x) − 3003.2, R^2 = 0.9115) in the range of 1 000−4 000 cells/mm^2. The quantification shows the potential of using BCECF-AM for measuring live cell concentrations directly in liquid samples by measuring the resonant

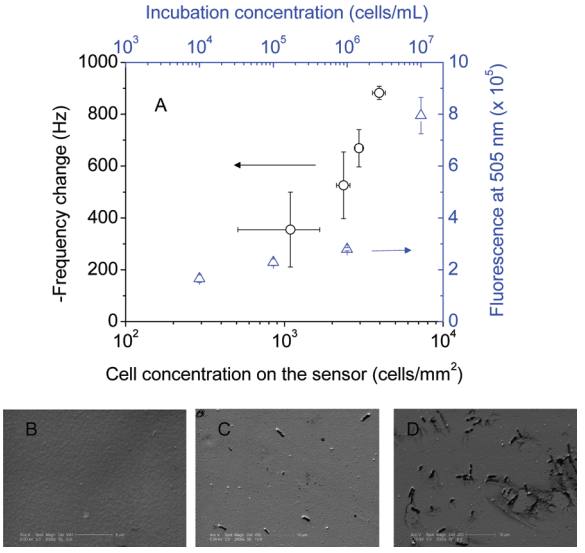


Figure 4. (A) Frequency response is compared with fluorescence response (505 nm) due to BCECF-AM injection. Sensor response to cell concentration on the surface (○) and fluorescence response to incubation concentration (Δ) are shown. Parts B−D are the SEM micrographs of the sensor surface after the associated experiments. They show sensor cell concentrations were 0, 1,090 ± 580, 3,960 ± 370 cells/mm^2 (n = 5) corresponding to 1 mL of 0, 10^4, and 10^7 cells/mL incubation, respectively. The scale bars in parts B, C, and D are 5, 10, and 10 μm, respectively.

frequency change since the cell concentration on the sensor surface is proportional to the sample concentration. The estimate of BCECF accumulation in each *E. coli* cell is found to be in the range of 0.15−0.22 fg. Considering each cell is ∼1 pg, the loading percentage of BCECF into *E. coli* cells is 0.015−0.022% of the cell mass. Since BCECF is fluorescent, we measured the fluorescence of incubated cells to confirm the sensor response. Experimentally, glass slides (cut to fit in a cuvette diagonally) were immobilized with *E. coli* following the approach used in sensor experiments and then was exposed to the same BCECF-AM (∼1.5 μM in 4 mL) for 1 h. The samples showed a higher fluorescence value with higher *E. coli* concentration and is in the same direction as the resonant frequency change (Figure 4). The increase of fluorescence intensity is particularly significant when the incubation concentration was increased to 10^6 and 10^7 cells/mL. This indicates accumulation of BCECF in viable *E. coli* cells and verifies the PEMC sensor response to BCECF-AM. The typical distribution of *E. coli* cells on the sensor surface is shown in parts B, C, and D of Figure 4, which are the SEM images after incubation in 1 mL of 0, 10^4, and 10^7 cells/mL samples, respectively. The sensor that was not incubated in *E. coli* was quite clean. A higher *E. coli* concentration yielded higher cell density on the sensor surface. For analysis of a pathogenic bacterium of interest, the antibody specific to the pathogen can be immobilized on the sensor surface to capture the desired target through antibody−antigen binding. The sensor can then be exposed to BCECF-AM for examining if the bound cells are viable using the method developed in this study. Thus, both quantification and viability information of a sample can be obtained with the approach described here, thus improving the TTR.

CONCLUSIONS

A cantilever biosensor in combination with BCECF accumulation can be used for detecting the viability of cells at a detection sensitivity of ∼2 000 *E. coli* cells. Because live cells respond to toxins, a live cell immobilized cantilever sensor would be useful in monitoring environmental toxic pollutants.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: (215) 895-2236. Fax: (215) 895-5837. E-mail: mutharasan@drexel.edu.

ACKNOWLEDGMENT

The authors are thankful for the financial support by the Environmental Protection Agency STAR Grant R833007.

REFERENCES

(1) Lazcka, O.; Campo, F. J. D.; Muñoz, F. X. *Biosens. Bioelectron.* **2007,** *22,* 1205–1217.
(2) Fu, Z.; Rogelj, S.; Kieft, T. L. *Int. J. Food Microbiol.* **2005,** *99,* 47–57.
(3) Perelle, S.; Dilasser, F.; Malorny, B.; Grout, J.; Hoorfar, J.; Fach, P. *Mol. Cell. Probes* **2004,** *18,* 409–420.
(4) Ashworth, M.; Horan, K. L.; Freeman, R.; Oren, E.; Narita, M.; Cangelosi, G. A. *J. Clin. Microbiol.* **2008,** *46,* 856–862.
(5) Lavrik, N. V.; Sepaniak, M. J.; Datskos, P. G. *Rev. Sci. Instrum.* **2004,** *75,* 2229–2253.
(6) Ziegler, C. *Anal. Bioanal. Chem.* **2004,** *379,* 946–959.
(7) Maraldo, D.; Rijal, K.; Campbell, G.; Mutharasan, R. *Anal. Chem.* **2007,** *79,* 2762–2770.
(8) Rink, T. J.; Tsien, R. Y.; Pozzan, T. *J. Cell Biol.* **1982,** *95,* 189–196.
(9) Wierda, W. G.; Mehr, D. S.; Yoon Berm, K. *J. Immunol. Methods* **1989,** *122,* 15–24.
(10) Heo, J.; Thomas, K. J.; Seong, G. H.; Crooks, R. M. *Anal. Chem.* **2003,** *75,* 22–26.
(11) Xu, S.; Mutharasan, R. *Anal. Chim. Acta* **2010,** *699,* 81–86.
(12) Chabot, V.; Cuerrier, C. M.; Escher, E.; Aimez, V.; Grandbois, M.; Charette, P. G. *Biosens. Bioelectron.* **2009,** *24,* 1667–1673.
(13) Ozkan, P.; Mutharasan, R. *Biochim. Biophys. Acta* **2002,** *1572,* 143–148.
(14) Campbell, G. A.; Mutharasan, R. *Anal. Chem.* **2007,** *79,* 1145–1152.
(15) Rijal, K.; Mutharasan, R. *Sens. Actuators, B: Chem.* **2007,** *124,* 237–244.
(16) Xu, S.; Mutharasan, R. *Sens. Actuators, B: Chem.* **2009,** *143,* 144–151.
(17) Detzel, A. J.; Campbell, G. A.; Mutharasan, R. *Sens. Actuators, B: Chem.* **2006,** *117,* 58–64.
(18) Fujikawa, H.; Kai, A.; Morozumi, S. *Food Microbiol.* **2004,** *21,* 501–509.