ARTICLE

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Sensitive and Selective Detection of Mycoplasma in Cell Culture Samples Using Cantilever Sensors

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ABSTRACT: In this article we report a new biosensor-based method that is more sensitive and rapid than the current approach for detecting mycoplasma in cell culture samples. Piezoelectric-excited millimeter-sized cantilever (PEMC) sensors respond to mass change via resonant frequency change. They are sensitive at femtogram level and can be used directly in liquid for label-free detection. Common cell culture contaminant, *Acholeplasma laidlawii* was detected in both buffer and cell culture medium. Two different sources (positive control from a commercial kit and ATCC 23206) were analyzed using antibody-immobilized PEMC sensor. Resonant frequency decrease caused by binding of *A. laidlawii* was monitored in real-time using an impedance analyzer. Positive detection was confirmed by a second antibody binding. The limit of detection (LOD) was lower than 10^3 CFU/mL in cell culture medium using PEMC sensor while parallel ELISA assays showed LOD as 10^7 CFU/mL. This study shows that PEMC sensor can be used for sensitive and rapid mycoplasma detection in cell culture samples.

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KEYWORDS: piezoelectric cantilever; mass change; *Acholeplasma laidlawii*; cell culture; ELSIA; limit of detection

Introduction

Mycoplasmas are the smallest free-living bacteria with a size of 300–800 nm. They lack a rigid cell wall and thus are resistant to common antibiotics that target cell wall synthesis. They have been found either attached to cell membrane or internalized within mammalian cells, causing compromised cell metabolic function. Among the over 100 species that have been discovered, about 20 species cause majority of cell culture contamination in both research laboratories and industrial processes (Rawadi and Dussurget, 1995). About 15–35% of continuous human and animal cell lines in current use are infected with mycoplasmas (Drexler and Uphoff, 2002). Earlier surveys showed as high as 65–80% incidence of mycoplasma infection of cell culture (Rottem and Barile, 1993). The six species, *Mycoplasma orale*, *M. hyorhinis*, *M. arginini*, *M. fermentans*, *M. hominis*, and *Acholeplasma laidlawii*, represent the majority of contaminating isolates (McGarrity, 1982; Uphoff and Drexler, 2002). The contamination causes alternations in growth rates, morphology, and cell viability. Both reduced antibody yield in hybridoma culture and total culture degradation have been reported. Therefore, routine assay of cell culture for possible contamination is an absolute requirement to ensure quality of biological products. The current industry standard for mycoplasma testing is specified in the Food and Drug Administration guideline (FDA, 1993).

Current method for detecting mycoplasma is by microbiological colony assay. Although this method is quite sensitive (1–10 CFU/mL), it requires a long incubation time (3–28 days) and experienced personnel for analysis (Masover and Becker, 1996). Polymerase chain reaction (PCR) is also sensitive (1–10 CFU/mL) for purified mycoplasma DNA, but its sensitivity deteriorates significantly to 10^3 CFU/mL in cell culture samples (Eldering et al., 2004; Rawadi and Dussurget, 1995; Tang et al., 2000; Wirth et al., 1994). Enzyme-linked immunosorbent assay (ELISA) is also used for routine mycoplasmas detection, but its sensitivity is poor (10^4–10^7 CFU/mL, depends on the species) (Garner et al., 2000). Both PCR and ELISA rely on culturing as a first step for increasing mycoplasma concentration and such a step often requires several days, particularly for low concentration samples. PCR method has shortened the time to results greatly (1–2 days) but has been reported to give false-positive/negative results for low counts ($<10^3$ CFU/mL) and is not readily implemented at production sites (Timenetsky et al., 2006). ELISA method is

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less expensive but requires a laboratory and an instrumentation arrangement. Because of its low sensitivity, a long incubation step becomes necessary. Therefore, there is a great need for a rapid, sensitive, selective, and inexpensive method for monitoring mycoplasma contamination in cell culture reactors.

Biosensors for cell culture systems have drawn a great deal of attention due to their rapid response, low cost, and high sensitivity (Campbell et al., 2007a; Jordan and Fernandez, 2008; Kintzios et al., 2006; Park and Shuler, 2003; Woolley et al., 2002). Among the biosensors that have been investigated, cantilever biosensors have been of special interest for the detection of biological analytes because of their ultra-high sensitivity and label-free detection (Singamaneni et al., 2008; Waggoner and Craighead, 2007). Details on cantilever sensor physics and operating principles are available in Ziegler (2004). For cantilever biosensors that operate in resonant (dynamic) mode, binding of target analyte to functionalized cantilever surface causes resonant frequency decrease (Thundat et al., 1994). Thus, one monitors resonant frequency during a sensing experiment.

We have reported on piezoelectric-excited millimeter-sized cantilever (PEMC) sensors that showed high mass-change sensitivity for the detection of a variety of pathogens and toxins. The analytes included *Escherichia coli* O157:H7 (Campbell et al., 2007b), *Bacillus anthracis* (Campbell and Mutharasan, 2007), staphylococcus enterotoxin B (SEB) (Maraldo and Mutharasan, 2007), and *Cryptosporidium parvum* (Campbell and Mutharasan, 2008). PEMC sensor is immune to damping effects in liquid and has been successfully used in sample flow arrangements. Mass-change sensitivity at levels of femtogram (fg, 10^{-15} g) was reported under sample flow conditions (Maraldo et al., 2007b). Such sensitivity is attractive for mycoplasma detection as mass of each organism is about 1 fg. In this study, we used antibody immobilized PEMC sensors for detecting the mycoplasma, *A. laidlawii*, as it is one of the most common contaminants in cell culture systems. To the best of our knowledge, in spite of a very active biosensor research over the past decade, no publication on detecting mycoplasmas has appeared in the literature. Detection experiments were conducted with readily available positive control samples from a commercial mycoplasma ELISA detection kit as well as with specially grown and calibrated *A. laidlawii* samples. Selectivity of sensor response was also evaluated in cell culture medium (5% serum) samples containing mammalian and bacterial cells.

Materials and Methods

Experimental Approach

Although a good number of antibodies for medically important pathogenic mycoplasmas are available, only a limited few are available for cell culture mycoplasma contaminants. An ELISA kit designed specifically for cell culture contaminants that recognizes the four species, *M. orale*, *M. hyorhinis*, *M. arginini*, and *A. laidlawii*, is available. The kit contains a coating polyclonal antibody (*c*pAb) and a biotinylated polyclonal antibody (*b*pAb) against each of the four species, and a positive control containing a mixture of the four species at undisclosed concentrations. In this work, we used both antibodies for detecting *A. laidlawii* in separate experiments. Since the sensor does not require a labeled antibody, we used either the *c*pAb or *b*pAb for confirming sensor response in a sandwich format. It should be mentioned that experiments were conducted with immobilized *c*pAb as well as immobilized *b*pAb.

The positive control in the commercial kit was used initially for developing data on detection feasibility and sensitivity. The manufacturer would not provide the composition of the positive control and thus we report the concentration as a volume of the positive control used. In order to obtain quantitative information on sensor limit of detection, we grew *A. laidlawii* cells and prepared a standard inactivated stock sample so that the same biological sample can be used for sensor response comparison studies. To ensure integrity of *A. laidlawii* sample, periodic ELISA assays using the commercial kit were carried out, and the results showed no variation over a 14-day period. Thimerosal inactivated cells were used in all PEMC experiments. For evaluating sensor performance, simultaneous measurements were carried out using the ELISA kit with both live and inactivated mycoplasma samples.

Reagents

Cysteamine, glutaraldehyde solution, streptavidin, and phosphate-buffered saline (PBS, 10 mM, pH 7.4) and others were purchased from Sigma–Aldrich (St. Louis, MO). Calibrated *A. laidlawii* (ATCC 23206, American Type Culture Collection, Manassas, VA) samples were prepared as described here. Mycoplasma-positive control, *b*pAb, and *c*pAb were from Roche kit (#11296744001, Roche Diagnostics GmbH, Penzberg, Germany). Antibody and antigen solutions were prepared in PBS (with 0.05% sodium azide) and stored at 4°C for use. Human epidermoid squamous carcinoma cells (A431) were obtained from another research laboratory at Drexel University and cultured in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Inc., Manassas, VA) supplemented with 5% fetal bovine serum and grown at 37°C in 5% CO_2. *E. coli* JM101 cells were grown in nutrient broth, killed in 1% Clorox®, centrifuged, and resuspended in PBS. Concentration was determined using a hemocytometer after staining with methylene blue.

Fabrication of PEMC Sensor

A PEMC sensor consists of two layers: a PZT layer (lead zirconate titanate, Piezo Systems, Woburn, MA) and a

quartz layer (SPI, West Chester, PA), bonded by a non-conductive adhesive. Details of fabrication are available in a previous publication (Maraldo et al., 2007b). Two PEMC designs were used. One is a flush design with a 2.7 mm × 1.0 mm × 0.127 mm ($l \times w \times t$) PZT bonded to 2.0 mm × 1.0 mm × 0.160 mm quartz. The second is an overhang design in which a 4.5 mm × 1.0 mm × 0.127 mm PZT bonded a 4.0 mm × 1.0 mm × 0.160 mm quartz. In both designs, the PZT end was anchored at one end. In the flush design, the glass layer was attached at the free-end with zero protrusion. In the overhang design, the glass layer protruded from the free-end by 1.0 mm. Wires (30 gauge) were soldered to electrodes of PZT layer and epoxy fixed in 6 mm glass tubing. The sensor was polyurethane (Wasser, Auburn, WA) spin-coated for providing electrical insulation. The two designs were shown to exhibit similar mass-change sensitivity (Maraldo et al., 2007b; Rijal and Mutharasan, 2007).

PEMC Sensor Surface Functionalization and Experimental Procedures

Prior to immobilization, 2.0 mm^2 (flush design) or 1.8 mm^2 (overhang design) of the PEMC sensor tip surface was freshly sputter-coated with 100 nm gold in a Desk IV sputtering system (Denton Vacuum, Moorestown, NJ). Two immobilization methods illustrated in Figure 1 were used in the experiments. In the first method (Fig. 1A), the sensor was incubated in 2 mM cysteamine in PBS overnight at room temperature, and then immersed in 2.5% glutaraldehyde solution for 1 h followed by 1 h incubation in 100 μg/mL anti-*A. laidlawii*. The sensor was rinsed with DI water and installed in a custom-made flow cell apparatus. In the second method (Fig. 1B), sensor was incubated in 100 μg/mL streptavidin solution for 2 h, followed by 1 h in 100 μg/mL *b*pAb prior to installation in the flow cell. The detection experiments were done at sample flow rate of 0.5 mL/min in an incubator maintained at 28 ± 0.1°C.

Sensor Calibration

Mass-change sensitivity of PEMC sensor was determined by depositing known mass of paraffin wax on the sensor surface and measuring the resulting change in resonant frequency after solvent evaporation (Campbell and Mutharasan, 2007). All measurements were made at 28 ± 0.1°C. Sensor surface was deposited with known wax mass using a stock paraffin wax in hexane solution prepared at concentrations ranging from 0.3 pg/μL to 100 ng/μL.

A. laidlawii Culture and ELISA Detection

A. laidlawii culture and ELISA detection were done as per vendor supplied protocol. In Supplementary Information we include detailed descriptions on: (1) *A. laidlawii* propagation and enumeration, (2) inactivation of

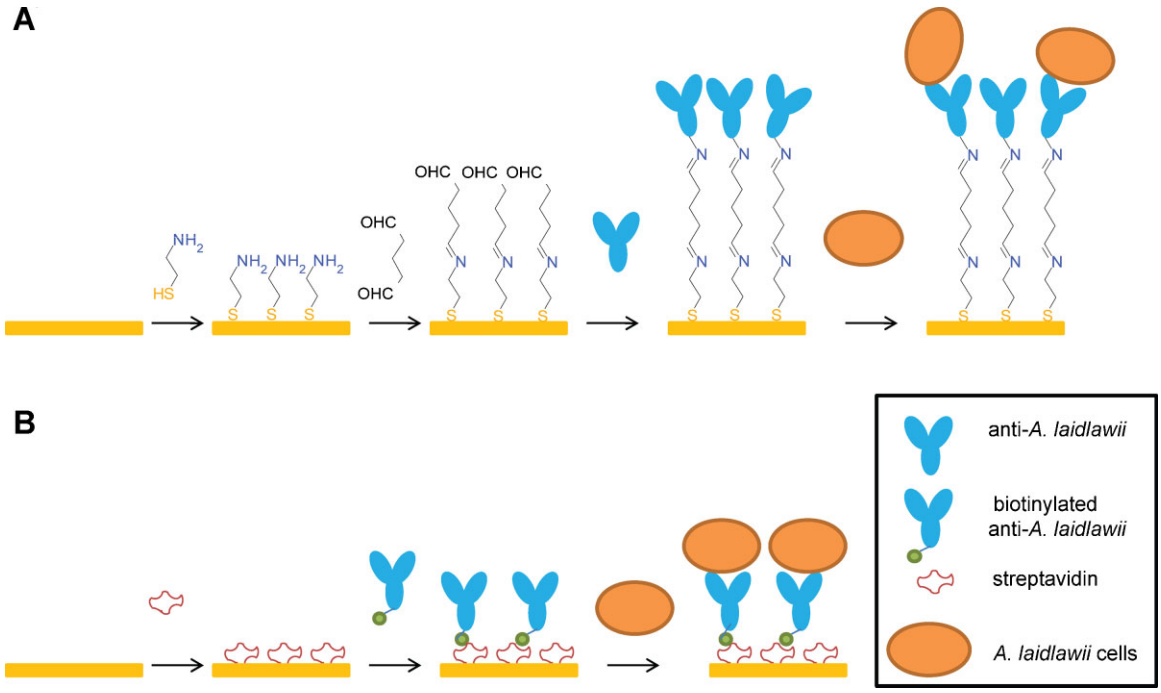

Figure 1. Illustration of antibody immobilization and immunoassays with PEMC sensor. **A**: Surface functionalization using cysteamine/glutaraldehyde chemistry on gold surface. **B**: Gold surface directly modified with streptavidin, and then biotinylated antibody was attached to the sensor surface via streptavidin/biotin binding. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

A. laidlawii cultures with thimerosal, and (3) ELISA detection of *A. laidlawii*.

Results and Discussion

PEMC Sensor Spectra and Sensitivity Calibration

The PEMC sensors used in this study had a resonant frequency near ~900 kHz. A number of fabricated sensors ($n = 23$) gave similar resonant frequency values within ±30 kHz of 900 kHz and were used in all detection experiments. As shown in Figure 2A, the resonant frequency decreases from 924.75 kHz in air to 856.50 kHz in DI water ($\Delta f = 68.25$ kHz) due to density increase of fluid surrounding the sensor. Although *Q*-factor (ratio of resonant frequency to the frequency width at half peak height) decreased from 40.3 to 29.5, it was sufficiently high for detecting resonant frequency within ±20 Hz in stagnant or flowing liquid samples.

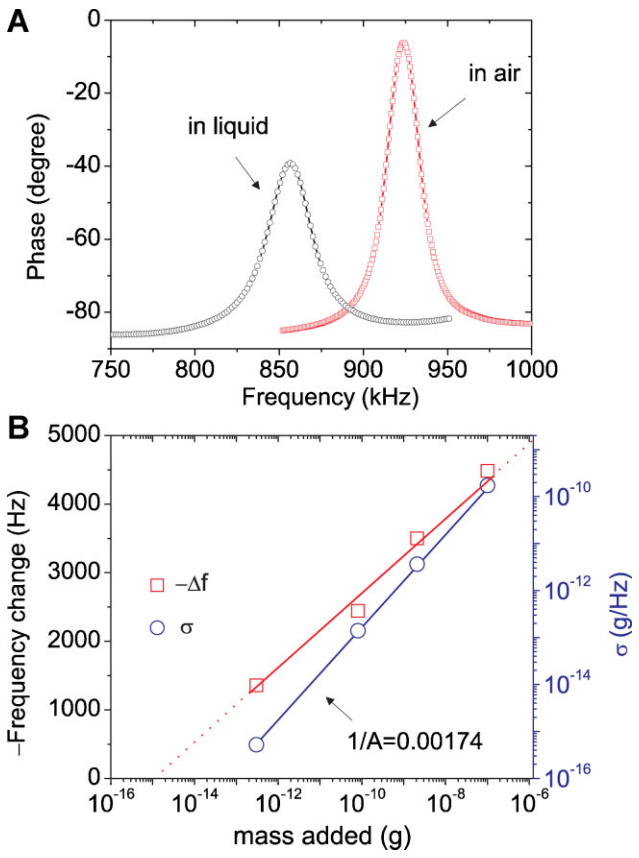

Figure 2. Typical sensor spectra and sensitivity calibration curve. **A**: Typical sensor spectra in air (□) and in DI water (○), respectively. **B**: Sensitivity calibration using diluted paraffin wax solutions. Sensor response ($-\Delta f$) is plotted as a function of log (mass added) (□), and log (σ) is plotted as a function of log (mass added) (○), respectively. PEMC sensor response to mass addition is log-linear and is similar to previous results. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Mass-addition (Δm) and resonant frequency change ($-\Delta f$) of a PEMC sensor is given by the following semi-log relationship (Maraldo and Mutharasan, 2007):

$$(-\Delta f) = A \log(\Delta m) + B \tag{1}$$

where A and B are constants and depend on sensor geometry. In principle, the parameter B should be ~0, but in practice we find it to have a non-zero intercept. Sensitivity defined as mass that causes unit resonant frequency change and can be determined from Equation (1) as

$$\sigma = \frac{\partial(\Delta m)}{\partial(\Delta f)} = \frac{\Delta m}{A} \tag{2}$$

where σ is the sensitivity of PEMC sensor at mass-addition (Δm). Since A is a constant, σ depends linearly on the magnitude of Δm. The smaller the value of Δm, the more sensitive the sensor is for a given sensor parameter A. Lower σ value indicates a higher mass-change sensitivity. Parameter σ is the mass of analyte that causes a unit resonant frequency decrease.

A typical sensitivity calibration result of flush design sensor is shown in Figure 2B, where resonant frequency response ($-\Delta f$) and $\log(\sigma)$ are plotted as function of log (mass added). The log-linear relationship between added mass and frequency response ($R^2 = 0.99$) confirms the validity of Equation (1). The parameters are $A = 573.9$ Hz and $B = 8442.2$ Hz, with ($-\Delta f$) in Hz and Δm in g. Sensitivity calculated using Equation (2) is $\sigma = 5.23 \times 10^{-16}$, 1.40×10^{-13}, 3.62×10^{-12}, and 1.75×10^{-10} g/Hz corresponding to the cumulative mass loading of 0.3 pg, 80.3 pg, 2.08 ng, and 102 ng, respectively. One notes in Figure 2B that as added mass increased, the sensitivity becomes numerically larger, indicating a decrease in mass-change sensitivity. This is an intrinsic characteristic of PEMC sensor response and is quantitatively given by Equation (1).

At the lowest added mass (300 fg) in the calibration experiments, the sensitivity was 5.23×10^{-16} g/Hz. If the noise level is ~20 Hz and we require a signal-to-noise ratio to be greater than 3, then a 60 Hz response would be caused by the attachment of ~30 fg. In liquid, resonant frequency change of PEMC sensor is directly attributable to mass change because the Reynolds number (Re) of PEMC at ~900 kHz is ~10^6. That is, the sensor responses are due to inertial effects and not due to viscous forces. Re is given as $(2\pi f b^2 \rho/\eta)$; b is cantilever width, f is resonant frequency, ρ and η are density and viscosity of fluid (Sader, 1998). If Re is low (~1), the dominant forces on the sensor are the viscous forces exerted by the surrounding liquid. Viscous damping not only reduces the quality factor but also significantly reduces sensitivity to changes in mass (Waggoner et al., 2009). In an earlier study we showed mass-change sensitivity in liquid decreased by 3–5 times compared to in air using two calibration methods; namely a wax deposition method

and bovine serum albumin (BSA) binding assay (Maraldo et al., 2007b). The difference in sensitivity is due to the difference in surface geometries; for example, wax forms a uniform film, while bound BSA on the surface is distributed non-uniformly. Nevertheless, having a means to relate mass-change response in air gives us a reasonable framework for interpreting resonant frequency changes in liquids.

Detection of Mycoplasma-Positive Control and Calibrated Samples in PBS

Sensor responses to sequentially increasing concentration of *A. laidlawii* in PBS were first obtained (Fig. 3A). We used the mycoplasma-positive control directly from Roche mycoplasma detection kit prepared in PBS. Since the positive control contains four species, the sample analyzed contained the competing species and the antibody immobilized on the sensor recognizes only *A. laidlawii*. The sensor was functionalized with *c*pAb via cysteamine–glutaraldehyde method in batch prior to the detection experiments. The sensor was installed in flow cell and resonant frequency was allowed to reach steady state with PBS as running buffer. Volumes of mycoplasma-positive control sequentially introduced were 1, 10, and 50 μL. At least 10 min steady-state time was observed prior to the addition of a higher dose. The resonant frequency decreases resulting from mycoplasma-positive control injections were 528, 606, and 525 Hz, respectively. The main responses for various doses were observed within the first 10 min after the sample was injected.

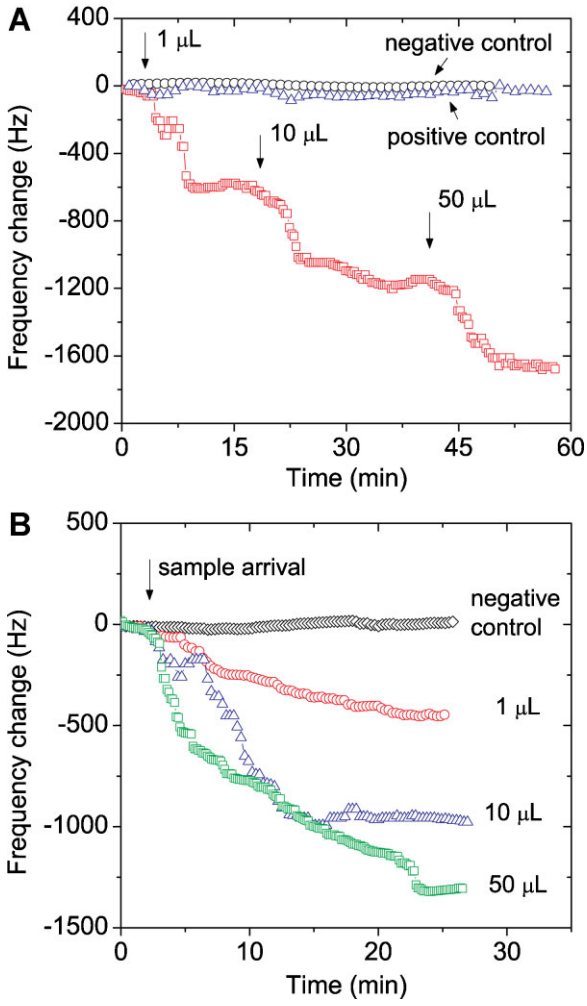

Figure 3. Detection of positive control in PBS. **A**: Response to sequential addition of mycoplasma-positive control from Roche kit. PEMC sensor was exposed to 1, 10, and 50 μL positive controls. Sequential decreases of 528, 606, and 525 Hz were obtained. **B**: Response to injections of 1, 10, and 50 μL positive control using fresh sensors in each experiment. The responses were 446, 997, and 1,304 Hz, respectively. The injected samples were diluted by 1:4 in flow loop. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Prior to conducting the detection experiments, responses to both positive and negative controls were obtained with the same sensor. Buffer control, which consisted of exposing unfunctionalized sensor to running buffer produced no response. In a separate set of experiments, unfunctionalized sensor was exposed to positive control (100 μL) and yielded a very small response of -50 ± 15 Hz, which is slightly higher than the noise level. In a negative control experiment, the sensor was immobilized with anti-*A. laidlawii*, and the introduction of *A. laidlawii*-free PBS gave a small response of -37 ± 4 Hz. Comparing the detection responses with the three controls, we conclude the decrease of sensor resonant frequency was due to the binding of *A. laidlawii* cells to the sensor surface.

Besides the sequential addition of various positive control sample volumes, experiments of single volume injection to freshly prepared sensor were also carried out and a set of typical responses is shown in Figure 3B. For separate injection of 1, 10, and 50 μL, the responses were 446, 997, and 1,304 Hz, respectively. The correlation of sensor ($n = 10$ for sensors used) responses to mycoplasma-positive control in PBS ($n = 3$–5 at each separate volume) is shown in Figure 4. Since the exact *A. laidlawii* cell numbers in the positive control is not known, we plot the sensor response as a function of volume (μL) added. One notes that the sensor response exhibits lower sensitivity beyond 35 μL, which may indicate sensor saturation. That is, the available antibody sites on the surface are not sufficient for the detection of a large number of cells. As seen in the inset, the response was plotted as a log function of volume added, which yielded $-\Delta f = 758.79 \log(C) + 174.78$ ($R^2 = 0.96$). The mycoplasma detection kit manual indicates a detection limit of *A. laidlawii* as 10^4–10^5 CFU/mL. It is estimated that in ELISA the positive control contains two to three orders higher concentrations of *A. laidlawii* cells than detection limit value. Based on this approximation, we estimate the cell number in 1 μL sample as $\sim 10^3$–10^5. As seen in Figure 3, the response to 1 μL sample was easily measurable. Since the sensor was not limited by sample volume, we expect detection sensitivity to be far better than 10^3–10^5 *A. laidlawii* cells.

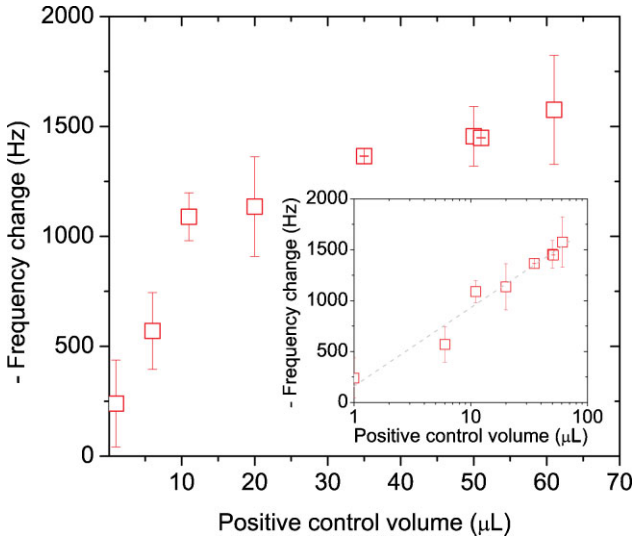

Figure 4. Resonant frequency change as a function of positive control volume injected. Inset shows resonant frequency response as a log function of volume injected. PEMC sensors exhibit log-linear relationship for mycoplasma-positive control detection. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

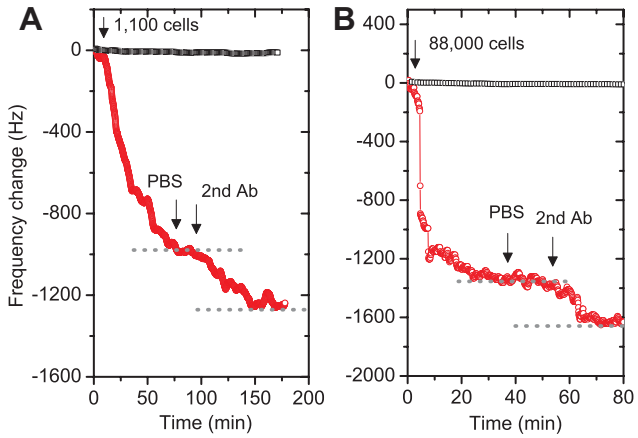

Figure 5. Detection of *A. laidlawii* in PBS and confirmation by a second antibody binding response. **A**: The PEMC sensor was exposed to 1,100 cells *A. laidlawii* (effective concentration 275 CFU/mL) for 50 min, then stabilized in PBS for 30 min before injection of 500 μL polyclonal antibody from Roche kit. The initial attachment of *A. laidlawii* gave a frequency decrease of 895 Hz, and the second antibody caused a further decrease of 256 Hz. **B**: The frequency response for injection of 88,000 cells (effective concentration 22,000 CFU/mL) was 1,512 Hz. After rinsing with PBS, a further 336 Hz decrease was obtained for 500 μL second antibody binding reaction. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Detection of Calibrated *A. laidlawii* in PBS and Cell Culture Medium

After accumulating good detection responses using positive control from the commercial kit, detection experiments with calibrated *A. laidlawii* cell samples were carried out. Spiked samples tested were in the concentration range of 10^3–10^7 CFU/mL since the ELISA data showed detection limit as 10^7 CFU/mL.

In Figure 5, sensor responses to samples containing 1,100 and 88,000 *A. laidlawii* cells are shown. Introduction of the calibrated samples caused decrease in resonant frequency due to *A. laidlawii* binding. Injection of 1,100 cells caused a decrease of 895 Hz, as shown in Figure 5A. Since the volume of flow loop was ~4 mL, the effective concentration was diluted to 275 CFU/mL. In order to confirm that the response was due to *A. laidlawii* binding, 500 μL *c*pAb was introduced into the flow loop after a PBS rinse. The logic here is that the polyclonal antibody will bind to the *A. laidlawii* cells that were already bound to the first antibody immobilized on the sensor surface (De Palma et al., 2007; Teramura and Iwata, 2007). Response to the antibody was strong and showed a decrease of 256 Hz, confirming the first sensor response was due to *A. laidlawii* binding. It is interesting to point out that the second antibody binding is akin to a sandwich assay in ELISA. Unlike in ELISA, both the first and second antibody contribute to detection signal and the second antibody provides for higher stringency. In a similar experiment shown in Figure 5B, PEMC sensor was exposed to 88,000 calibrated *A. laidlawii* cells (effective concentration 22,000 CFU/mL) in the same manner. The injection of *A. laidlawii* cells caused 1,512 Hz resonant frequency decrease. A further 336 Hz decrease was observed with the introduction of 500 μL *c*pAb. These two examples show the detection of *A. laidlawii* in PBS at as low a concentration as ~10^3 CFU/mL is feasible using PEMC sensors.

Since mycoplasma species are common contaminants in mammalian cell cultures, direct and rapid detection in cell culture containing serum without any pre-treatment is useful for evaluating practical performance of PEMC sensors. A detection experiment using PEMC sensor in cell culture medium is shown in Figure 6B. Sensor was functionalized with *b*pAb and stabilized in PBS. Cell culture medium containing serum (DMEM with 5% fetal calf serum, FCS) and A431 cells (5×10^6 CFU/mL; human skin cells) was introduced into flow loop in a one-through fashion so as to displace the flow loop with the new medium. A decrease of 330 Hz in frequency was observed due to higher density of the cell culture medium and was verified in a separate experiment (see Fig. 6A). After sensor stabilized in the cell culture medium, 1 mL killed *E. coli* JM101 (10^7 CFU/mL) cells were introduced into the flow loop in recirculation mode. Although one sees an increase in noise level in resonant frequency, no significant decrease in resonant frequency occurred. After steady state was reached, the circulating fluid was DMEM-serum-A431 (5×10^6 CFU/mL) and *E. coli* JM101 (2.5×10^6 CFU/mL), which simulated a strongly contaminated cell culture medium environment. The absence of resonant frequency responses indicates that neither A431 nor *E. coli* JM101 attached to the sensor surface. In this background, *A. laidlawii* detection experiments were conducted. At 40 min, 1 mL of 1,000 CFU/mL *A. laidlawii* (effective concentration 250 CFU/mL in flow loop) prepared in cell culture medium (DMEM with 5% FCS) was

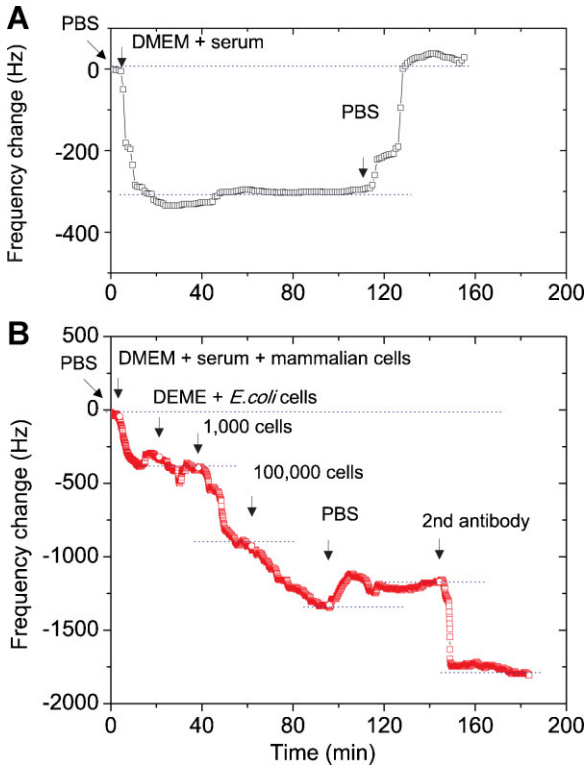

Figure 6. **A**: PEMC responds to density when DMEM was introduced to replace the running buffer PBS; resonant frequency decreased by 315 Hz due to density. Upon replacing DMEM with PBS, the resonant frequency fully recovered back to the original value. **B**: Detection experiment in DMEM containing serum (5% FCS), mammalian cells (5×10^6 cells A431 cells), and *E. coli* JM101 cells (10^7 cells) as background. The PEMC sensor was functionalized with antibody prior to experiment. 692 Hz decrease was observed for 1,000 *A. laidlawii* cells, and a further 428 Hz decrease was seen for 100,000 cells. Second antibody binding gave a further decrease of 575 Hz. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

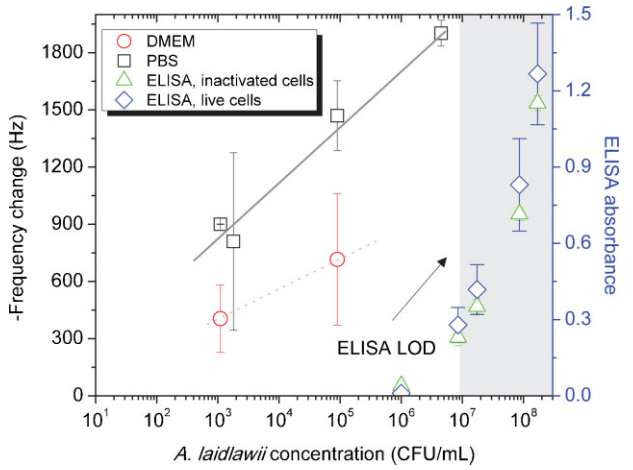

Figure 7. Comparison of PEMC and ELISA results. For PEMC sensor, resonant frequency response is plotted as a log function of *A. laidlawii* concentration with a detection limit of 10^3 CFU/mL in the dynamic range of 10^3–10^7 CFU/mL. Measurements in PBS (□) consistently gave higher response in comparison to cell culture medium (○). Detection limit by ELISA assay was 10^7 CFU/mL for both live cells (◇) and inactivated cells (Δ). [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

introduced into the flow loop. Note that the circulating fluid and the *A. laidlawii* sample were of the same composition except for the mycoplasma cells, and thus no density difference was introduced. Resonant frequency decrease of 692 Hz was observed shortly after sample introduction. At 62 min, 1 mL of 100,000 CFU/mL *A. laidlawii* was further injected in and an additional decrease of 428 Hz was observed. PBS flush caused a recovery of 215 Hz and was slightly less than the initial density-induced decrease of 330 Hz, which may suggest an incomplete PBS rinse. After stabilization in PBS, 500 μL of freshly prepared *c*pAb was introduced and a significant resonant frequency decrease of 575 Hz was observed. The second antibody binding response confirmed that the initial sensor response was indeed due to *A. laidawii* attachment. A similar experiment where the sensor was not exposed to *A. laidawii* sample did not result in a resonant frequency response.

Quantification of Sensor Response

It is useful to examine if the response of PEMC sensor to various concentrations of *A. laidlawii* can be quantified. Sensor responses to various concentrations of calibrated *A. laidlawii* samples in PBS ($n = 3$ or 4) and DMEM ($n = 3$) are given in Figure 7. First-order analysis indicates that the sensor response ($-\Delta f$) correlated well with *A. laidlawii* concentration (C_{Al}) with the relationship given in Equation (1). This relationship has been tested true in other detection experiments for a pathogen and a 30-kDa toxin (Campbell and Mutharasan, 2006; Maraldo and Mutharasan, 2007). For the case of *A. laidlawii* detection in PBS, the correlation was excellent ($-\Delta f = 310.14 \log(C_{Al}) - 77.212$, $R^2 = 0.98$). For experiments conducted in DMEM, sensor response was also proportional to *A. laidlawii* concentration. Comparing the results in PBS and DMEM, one notes that the response in DMEM was consistently ~50% lower than in PBS, which suggests that the sensor is ~50% less sensitive in complex serum/mammalian cells/*E. coli* background than in PBS. The decrease in response magnitude in complex medium has been observed for the detection of SEB and a cancer biomarker using PEMC sensor (Maraldo and Mutharasan, 2007; Maraldo et al., 2007a). Similar sensitivity decreases in complex matrix (serum or plasma) have also been reported in other biosensor systems (Cao et al., 2006; Cui et al., 2003; Hwang et al., 2009; Oshima et al., 2005). We attribute reduced sensor response to reduced access to binding sites, as well as possible loss of available antibody sites.

It is useful to examine the resonant frequency shifts obtained (Fig. 7) with the mass of *A. laidlawii* bound to the antibody on the sensor. For example, at 10^3 cells in sample, the response was ~900 Hz. If we ignore the loss of sensitivity in liquid and use the calibration data in Figure 2, the corresponding mass of target can be estimated as ~60 fg, or ~60 *A. laidlawii* cells. Since the response in liquid is lower by

about a factor 3–5, the actual number of cells attached can be estimated as ~180–300 cells. This is within the order of magnitude of the expected response. It is to be noted, while sensors can be calibrated in air with great accuracy, calibration in liquid is not easily accomplished. We use the estimates noted above to provide an approximate analysis of the sensor response using air calibration results.

Comparison of PEMC Sensor With ELISA

ELISA assays of thimerosal-inactivated and live *A. laidlawii* cells were within ~10–15%. The inactivated cells gave a slightly lower absorbance value compared to live cells. Since binding in ELISA assay was only marginally compromised by using inactivated cells, we can compare PEMC sensor responses with the ELISA results conducted with both live and inactivated cells, and the latter is included in Figure 7. As can be seen in Figure 7, detection limit of the freshly grown or thimerosal-treated *A. laidlawii* using ELISA was $\sim 10^7$ CFU/mL. On the other hand, the LOD of the PEMC sensor was lower than 10^3 CFU/mL, which is four orders of magnitude lower than ELISA LOD. The results suggest that PEMC sensors can significantly improve our ability to monitor contamination in cell culture systems. If one uses sample preparation steps such as centrifugation and resuspension in a buffer, a further improvement in LOD can potentially be achieved using PEMC sensors.

The FDA recommended positive control inoculation is 10–100 CFU/mL for testing cell culture samples. Although experiments at such a low concentration have not been carried out with PEMC sensors, it would be prudent to include a short incubation step to permit growth of contaminating mycoplasma cells prior to detection, as this will ensure detection of live cells. Because LOD is lower than 10^3 CFU/mL, the required incubation step would be shorter than that is required for plate-counting microbiological method or PCR approach. In a future report we will examine the applicability of the PEMC sensors at ultralow concentrations of 1–100 CFU/mL by using a second and probably a third antibody to amplify the sensor response.

Conclusion

In this study, we showed successful detection of *A. laidlawii* at 10^3 CFU/mL in both PBS and cell culture medium. Anti-*A. laidlawii* immobilized PEMC sensor showed positive responses for both positive control from the Roche mycoplasma detection kit and self-grown *A. laidlawii* cells. Detection of calibrated *A. laidlawii* samples showed positive response in the dynamic range of 10^3–10^7 CFU/mL in buffer medium. The PEMC sensor showed about four orders higher sensitivity compared with ELISA method conducted in parallel using the same *A. laidlawii* stock and the same antibody reagents. Furthermore, the time required for positive detection using PEMC sensor was less than 1 h, which is attractive for timely determination of mycoplasma contamination.

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