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Real-Time, Label-Free, All-Electrical Detection of *Salmonella typhimurium* Using Lead Zirconate Titanate/Gold-Coated Glass Cantilevers at any Relative Humidity

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Abstract

We have examined non-insulated PZT/gold-coated glass cantilevers for real-time, label-free detection of *Salmonella t.* by partial dipping at any relative humidity. The PZT/gold-coated glass cantilevers were consisted of a 0.127 mm thick PZT layer about 0.8 mm long, 2 mm wide bonded to a 0.15 mm thick gold-coated glass layer with a 3.0 mm long gold-coated glass tip for detection. We showed that by placing the water level at the nodal point, about 0.8 mm from the free end of the gold-glass tip, there was a 1-hr window in which the resonance frequency was stable despite the water level change by evaporation at 20% relative humidity or higher. By dipping the cantilevers to their nodal point, we were able to do real-time, label-free detection without background resonance frequency corrections at any relative humidity. The partially dipped PZT/gold-coated glass cantilever exhibited mass detection sensitivity, $\Delta m/\Delta f = -5 \times 10^{-11} \text{g/Hz}$, and a detection concentration sensitivity, 5×10^3 cells/ml in 2 ml of liquid, which was about two orders of magnitude lower than that of a 5 MHz QCM. It was also about two orders of magnitude lower than the infection dosage and one order of magnitude lower than the detection limit of a commercial Raptor sensor.

Keywords

PZT microcantilever; *In-situ* biodetection; humidity control; nodal point

I. Introduction

Current methods for pathogen detection in water include filtration culture methods,^{1,2} fluorescence-based methods,^{3–5} fluorescence probes,⁴ deoxyribonucleic acid (DNA) microarray methods,⁵ and others. These approaches do not allow *in-situ* measurements since they require multiple steps of labeling and purification before the final measurement by which time the captured pathogens are no longer in the initial liquid medium. On the other hand, silicon-based microcantilevers can be direct and do not require labeling. Antibody

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receptors are coated on the microcantilever surface to bind target DNA, protein molecules, or bacteria. ^{6–12} Binding of target antigens to the receptors on the microcantilever surface is detected by monitoring the microcantilever's tip bending displacement,^{6–10} or its resonance frequency shift,^{11–12} by an external optical system. However, silicon-based microcantilevers cannot withstand damping by liquid and exhibit low Q factors (the ratio of the resonance peak frequency to the resonance peak width at half peak height) in liquid,¹³ rendering them ineffective for in-liquid detection. Piezoelectric cantilevers, on the other hand, can self-excite and self-detect the mechanical resonance electrically. Although lead zirconate titanate (PZT) microfabricated cantilevers^{14–16} have been demonstrated for label-free biological detections, many detection measurements were done after the sensors were removed from the liquid and dried,^{14–16} or done by optical measurements.¹⁷ The reason that these sensors could not perform real-time, label-free, all-electrical detection was either they could not withstand liquid damping or there was no electrical insulation.^{14–16} Even with electrical insulation coating, charges, especially protons could still diffuse through the insulation layer to cause degradation of the resonance peak over time and a background resonance frequency shift due to charge penetration through the insulation layer over time. Therefore, a sensor that can perform real-time, label-free, and sensitive electrical detection without the need of electrical insulation is a tremendous advantage.

Earlier work showed that 3 mm long piezoelectric PZT/stainless steel cantilevers readily fabricated using commercially available PZT with a 5 mm long stainless steel tip at the free end could exhibit a good Q value¹⁸ and perform *in-situ* yeast cell detection,¹⁹ where the non-piezoelectric stainless steel tip served as the detection pad alleviating the need to insulate the PZT layer. At the time, the sensitivity of the sensor was such that the effect of water level due to the evaporation did not play an important role. However, with the detection sensitivity improved with a reduced cantilever size^{20–21} the effect of the water-level change due to evaporation could give rise to a background up-shift in the cantilever resonance frequency.²² Therefore, a predetermined background frequency shift needs to be subtracted from the detection result.²³ However, the background resonance frequency shift depends on the humidity level and may also depend on the dipping depth. Therefore this predetermined background correction is prone to errors associated with dipping depth variation and humidity level changes from day to day. Often the background correction became an adjustable parameter in the data analysis.

To minimize the effect of water level change due to evaporation on the background resonance frequency up-shift of a cantilever partially dipped in the liquid, one choice is to do the detection at high humidity, e.g., at 90% relative humidity such that there is no appreciable water level change to affect the cantilever's resonance frequency.²⁴ However, this obviously requires the detection be done in a humidity controlled environment or a measuring chamber, which is not always convenient. It is known that a piezoelectric cantilever with a non-piezoelectric tip exhibit a high second-mode resonance peak which can be utilized for in-liquid detection. Furthermore, the second-mode wave function exhibit a nodal point at about the mid point of the non-piezoelectric tip and moreover, because there is negligible vibrations at and near the nodal point, the cantilever is virtually insensitive to mass changes at the nodal point and exhibits a diminished mass detection sensitivity at the nodal point.²¹ Thus, one may alternatively explore utilizing this unique characteristic of a nodal point for detection with a partially dipped cantilever without the need to control the humidity.

In this study we will examine all-electrical *in-situ* *Salmonella t.* detection using a PZT/gold-coated glass cantilever with a nonpiezoelectric gold-coated glass tip dipped in a phosphate buffer saline (PBS) solution to cantilever's nodal point without humidity control and without insulating the PZT layer. The study consists of two parts. The first part is to investigate how

evaporation at different humidity levels affects the cantilever resonance frequency at various dipping depths in PBS to empirically identify the nodal point, which will also be confirmed by theoretical calculations. The second part of the study is to dip the nonpiezoelectric tip to the nodal point and carry out real-time, in-liquid *Salmonella t.* detection in PBS without insulation and without controlling the humidity. We will show that for all three humidity levels (20%, 50%, and 90%) investigated, the resonance frequency shifts upwards as a result of water evaporation and the rate of the second-mode resonance frequency up-shift exhibits a minimum around the nodal point of the second-mode vibration wave function. With the cantilever dipped to the nodal point (around 0.8 mm from the free end), a minimum of 1/2 hour window of less than 2 Hz/min resonance frequency up-shift could be achieved at any relative humidity 20%. This background resonance frequency up-shift by evaporation is different from the binding of *Salmonella* cells to the antibody-immobilized sensor surface which produces a down-shift in the resonance frequency due to the compressive stress in the adsorbed layer⁶ (or tensile stress in the cantilever) and the mass increase.^{19,20} By monitoring the sign of the frequency shift and using the nodal-point approach, we showed that the present PZT/gold-coated glass cantilever exhibited a concentration limit of 5×10^3 cells/ml in 2 ml of liquid for *Salmonella t.* detection.

II. Experimental Procedure

Two PZT/gold-coated glass cantilevers of similar dimensions were constructed by bonding a 127 μm thick PZT layer (T105-H4E-602, Piezo System Inc., Cambridge, MA) to a 150 μm thick gold-coated glass layer (Fisher Scientific, Pittsburgh, PA) using a nonconductive epoxy (Loctite, Rocky Hill, CT). Cantilever A had a PZT section 0.8 mm long, 2.0 mm wide and a 3.0 mm long gold-coated glass tip. Cantilever B had a PZT section 0.7 mm long, 2.2 mm wide and a 2.7 mm long gold-coated glass tip. As an example, Fig. 1(a) shows an optical micrograph of a typical PZT/gold-coated cantilever and Fig. 1(b) a schematic of the PZT/gold-coated glass cantilever structure, respectively. Cantilever A was used for all the experiments shown in Figs. 1–9 and 11 and cantilever B for the results shown in Fig. 10. The 150 nm thick gold layer was coated with 10 nm thick chromium as the bonding layer between the glass substrate and the gold layer. Both chromium and gold were deposited by e-beam evaporation (E-gun: 3kW, vacuum 2×10^{-6} torr) at the Microfabrication Laboratory of University of Pennsylvania. The gold-coated glass layer was extended at the free end to form a 3 mm long tip which was subsequently immobilized with antibodies for cells detection. The chemical used were glycine (Fisher Scientific, Fair Lawn, NJ), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (Sigma, St. Louis, MO), N-Hydroxysuccinimide (NHS) (Aldrich, Milwaukee, WI), HCl (Fisher Scientific, Pittsburgh, PA), and 3-mercaptopropionic acid (MPA) (Aldrich, Milwaukee, WI). The heat-killed *Salmonella t.* cells and the affinity purified antibody to salmonella common structural antigens were purchased from Kirkegaard & Perry Laboratories, Inc. Gaithersburg, MD. After each detection, the cantilever was dipped in a mixed solution of glycine and HCl at pH = 2.2 for 30 min to release the cells. After the release in the glycine/HCl solution, the antibody-immobilized cantilever sensor was reused for detection. With this glycine/HCl release procedure, each cantilever sensor was reused for more than 20 detections. The scanning electron microscopy (SEM) micrographs of the cells detected at the gold-coated cantilever tip were obtained using FEI/Phillips XL30 (FEI Company, Hillsboro, Oregon). The detection was carried out by monitoring the resonance frequency of the cantilever using an impedance analyzer (Agilent 4294A, Agilent, Palo Alto, CA). The mass detection sensitivity of the gold-coated glass cantilever sensors was calibrated with a 5 MHz AT-cut quartz crystal microbalance (QCM) (QCM100, Stanford Research Systems, Inc. Sunnyvale, CA). The resonance frequency of the QCM was also monitored by the Agilent 4294A impedance analyzer.

III. Results and Discussions

III.1. Cantilever resonance spectrum characteristics

Figure 2(a) shows an in-air phase-angle-versus-frequency resonance spectrum of a PZT/gold-coated glass cantilever (dashed line). Off resonance, the cantilever behaved as a capacitor exhibiting a phase angle of -90° . At resonance, the flexural motion gave rise to a peak in the real part of the electrical impedance, and hence a peak in the phase angle. The spectrum shows that below 100 kHz, the cantilever exhibited two resonance peaks, the first peak at 24.8 kHz and the second peak at 76.7 kHz, both with quality factor, $Q = 45$ where Q is defined as the ratio of the resonance frequency over the peak width at half the peak height. Also shown in Fig. 2(a) are the calculated resonance frequencies obtained as vertical lines using the method describe in Ref. 21. As can be seen, both peak positions agreed with the theoretical calculation. Furthermore, the second-mode wave form exhibited a nodal point near the free end. As can be seen from Fig. 2(b) where the theoretical first and second resonance wave forms calculated using the theory in Ref. 21 are shown together with a schematic of the side view of the cantilever. The theoretical nodal point was at $x = 0.8$ mm where x was measured from the free end of the tip.

III.2. Nodal Point location and rate of background resonance frequency shift characterization

In all detections, we only dipped the lower portion of the gold-coated glass tip in the solution as schematically shown in Fig. 3(a). The resonance spectrum of the cantilever with a partially dipped gold-coated glass tip (dipping depth 0.8 mm) is also shown in Fig. 2(a) (solid line). As can be seen, the resonance frequencies of the cantilever decreased when its gold-coated glass tip was partially immersed in water. In Fig. 3(b) we plot the cantilever resonance frequency versus the dipping depth, x . As can be seen, the resonance frequency decreased with an increasing dipping depth, x . The decrease in the cantilever's resonance frequency in PBS is mainly due to the mass of the surrounding liquid that moved in phase with the cantilever, which increased the effective mass of the cantilever.¹⁹ However, the in-PBS resonance frequency peaks still retained the high Q values with $Q = 30$ for the first peak, and $Q = 35$ for the second peak in PBS.

Since the resonance frequency depended on the dipping depth and evaporation of water could decrease the cantilever dipping depth over time, background resonance frequency up-shift could arise from the evaporation of water during detection. The background resonance frequency up-shift due to evaporation was investigated at three different relative humidity levels, 20%, 50%, and 90%. The 20% relative humidity was the typical ambient humidity level in the laboratory in the winter season. The 50% and 90% relative humidity levels were achieved and maintained by blowing water vapor with a humidifier (model 701, Sunbeam Product, Inc. Boca Raton, FL) into a semi-closed 126-liter glove box (model H500280000, Bel-Art Products, Pequannock, NJ). The humidity and temperature were monitored using a humidity and temperature sensor (model 35519-050, VWR International, West Chester, PA). The temperature of all three humidity levels was 21 ± 0.5 °C. The uncertainty of the relative humidity levels was $\pm 4\%$ relative humidity for 20% and 50% relative humidity and $\pm 10\%$ for 90% relative humidity as the humidity sensor could only measure the humidity accurately below 90% relative humidity. To determine the rate of water level change, we filled a small container of less than 2 ml capacity with the phosphate buffer solution (PBS) (the medium of all detections) and placed it in each relative humidity for 8–24 hr and measured the resultant water level change. The obtained rates of water level change at 20% and 50% humidity were 0.3 and 0.16 mm/hr, respectively. For the 90% relative humidity we did not observe a measurable water level change over a period of 24 hr partly due to the high

humidity level and partly due to the uncertainty in humidity control from the limitation of the humidity sensor as mentioned above.

To examine the effect of relative humidity on the cantilever's background resonance frequency shift at each of the three humidity levels, we dipped the cantilever in the PBS at different dipping depths and monitored the cantilever's second-mode resonance frequency for 30 min at each dipping level. It should be noted that because the dipping depth had a one-to-one correlation with the resonance frequency (see Fig. 3) positioning the water level at a certain depth was simply done by adjusting the dipping depth *until the right resonance frequency was obtained*, which took less than 15 sec. This was different from using distance as the criterion, which might be difficult as many dipping depths were smaller than the meniscus of water. Using a pre-determined resonance frequency as criterion, dipping the cantilever to a certain depth was straightforward, rapid (in less than 15 sec), and highly repeatable. Consequently, the resultant detection results were also highly repeatable and we have repeated this practice more than 40 times to obtain all the detection results in this paper. As we will show below, resonance frequency shift of separate detections of the same detection conditions exhibited an error bar of less than 20%.

To show the different rate of resonance frequency shift at a different dipping depth, we show in Fig. 4(a)–4(e) the resonance frequency versus time at 20% relative humidity at $x = 0.35, 0.6, 0.8, 1.1, \text{ and } 1.6$ mm, respectively. The initial resonance frequency was 70, 68.2, 66.4, 59.8, and 51.3 kHz at $x = 0.35, 0.6, 0.8, 1.1, \text{ and } 1.6$ mm, respectively. From Figs 4(a)–4(e), one can see that the minimal increase in resonance frequency with time occurred at $x = 0.8$ mm (Fig. 4(c)) at a rate of about 2 Hz/min.

The rate of resonance frequency up-shift, $\Delta f/\Delta t$ versus x at 20%, 50%, and 90% relative humidity is shown in Fig. 5(a) with $\Delta t = 30$ min and Δf denoting the corresponding frequency shift. As can be seen, at 90% relative humidity, $\Delta f/\Delta t$ was zero at most x except near the free end. At each x , $\Delta f/\Delta t$ increased with a decreasing relative humidity, indicative that these resonance frequency shifts were related to water level changes due to evaporation. Furthermore, the curves for both 20% and 50% relative humidity exhibited a minimum in $\Delta f/\Delta t$ close to zero at $x = 0.8$ mm, the nodal point. For comparison, we calculated the theoretical second-mode $-\Delta f/\Delta m(x)$ versus x using the theory described in Ref. 21 and plotted the result in Fig. 5(b) where $\Delta m(x)$ is the local mass change at x and Δf is the corresponding frequency shift. The theoretical $-\Delta f/\Delta m(x)$ exhibited a minimum at the nodal point, $x = 0.8$ mm and that for a given mass change, a much larger resonance frequency shift was obtained away from the nodal point. To further illustrate that the experimental curves of $\Delta f/\Delta t$ shown in Fig. 5(a) were indeed due to the combination of evaporation and the effect of the nodal point, we plot the ratio $(\Delta f/\Delta t)/(-\Delta f/\Delta m(x))$ versus x in Fig. 5(c). Because $\Delta f/\Delta m(x)$ was zero at the theoretical nodal point, $x = 0.8$ mm, any uncertainty in dipping depths and $\Delta f/\Delta t$ measurements near or at the nodal point could cause huge errors in the $(\Delta f/\Delta t)/(-\Delta f/\Delta m(x))$. To avoid such errors, in Fig. 5(c), we only included data away from the nodal point such that the ratio $(\Delta f/\Delta t)/(-\Delta f/\Delta m(x))$ was meaningful. Note that the ratio $(\Delta f/\Delta t)/(-\Delta f/\Delta m(x))$ had a unit of g/min and should be proportional to the evaporation rate, which according to the Langmuir's Equation²⁵ should be proportional to (1-relative humidity) given that the temperature was constant. As can be seen in Fig. 5(c), $(\Delta f/\Delta t)/(-\Delta f/\Delta m(x))$ were by and large independent of x and fell into three separate horizontal lines as indicated by the dashed lines. From Fig. 5(c), a ratio of the $(\Delta f/\Delta t)/(-\Delta f/\Delta m(x))$ at 20, 50 and 90% relative humidity can be roughly estimated as 9:4:1, which was within the experimental uncertainty of the ratio of (1-relative humidity), 8:5:1, validating that the background resonance frequency up-shift was indeed due to water evaporation and that at the nodal point, the rate of the background resonance frequency shift vanished. Furthermore, as can be seen from Fig. 5(a), at 20% humidity, the rate of resonance frequency could remain at less

than 2 Hz/min for about 1/2 hr. i.e., corresponding to a dipping depth window of about 0.15 mm. At 50% and 90% humidity, because the evaporation rate is much lower, the time interval and the dipping depth window at which minimal background resonance frequency shifts occurred were even larger.

The above results indicated that with a controlled 90% relative humidity, virtually no background resonance frequency up-shift occurred at $x = 0.8$ mm, which could permit detection without a background resonance frequency shift as demonstrated by Zhu et al. 24. However, to do detection at 90% relative humidity requires a humidity controlled chamber. Without relative humidity control, as can be seen from the above result, there is a 1/2 hour window with less than 2 Hz/min background resonance frequency up-shift at 20% relative humidity or higher. This indicates that with a dipping depth of 0.8 mm, there would be minimal background resonance frequency up-shift of less than 2 Hz/min for at least 1/2 hour at any humidity level higher than 20% relative humidity. Since most of the ambient relative humidity levels were larger or equal to 20%, it followed that with the water level placed at the nodal point, we had at least one hour of time in which the background frequency shift was at most +2 Hz/min or +60 Hz for 30 min. All the following detection experiments were carried out with the water level placed at the nodal point without humidity control. To be conservative, we did not perform any correction due to the background up-shift. If corrections were done, the magnitudes of the negative frequency shifts due to detection should actually be larger than we reported below.

III. 3. Antibody Immobilization and mass detection sensitivity calibration

The gold-coated glass tip was first cleaned with a mixture of 1 part H_2O_2 and two parts 98% H_2SO_4 for 5 min and rinsed by distilled water. The cleaned gold-coated tip was then dipped in a 2-mM MPA solution for 3 hr to form a self-assembled monolayer. The MPA-coated tip was then soaked in a solution containing 5 mg/ml of EDC and 5 mg/ml of NHS for 30 min to activate the carboxyl group to form an amide. After rinsing with distilled water, the tip was dipped in a 400 nM antibody solution for 30 min to immobilize the antibody on the cantilever tip by the reaction of the amide with a primary amine of the antibody to result in a peptide bond. The above immobilization procedure was taken from references.^{26,27} In Figs. 6(a) and 6(b), we plot the resonance frequency shift versus time for the first 30 min of NHS modification and antibody immobilization, respectively (full circles). The two procedures were also carried out with a 5 MHz QCM with a gold electrode without flow. The resultant resonance frequency shifts versus time from the QCM were also plotted in Figs. 6(a) and 6(b) (open squares). Figure 6(a) shows that the NHS modification resulted in a down-shift of about -90 Hz in 30 min whereas the same procedure did not result in a discernable resonance frequency shift with QCM. In Fig. 6(b), the antibody immobilization at the cantilever tip resulted in a rapid down-shift in the cantilever's resonance frequency shift in the first 6–7 min and saturated at a -450 Hz down-shift after 30 min. The same procedure yielded a frequency down-shift of -45 Hz in 30 min with QCM. By simply comparing the resonance frequency shifts, the current PZT/gold-coated glass cantilever was about 10 times more sensitive than a 5 MHz QCM.

From the above QCM measurement, the mass change per unit area Γ at the gold-coated cantilever tip due to the antibodies immobilization can be deduced by the Sauerbrey equation^{28–30}

$$\Delta f_{\text{QCM}} = \frac{-2f_{\text{QCM}}^2}{\sqrt{G\rho}} \Gamma, \quad (2)$$

where Δf_{QCM} is the resonance frequency shift of QCM, G and ρ the shear modulus and the density of quartz, respectively, f_{QCM} the resonance frequency of the QCM. With $f_{\text{QCM}} = 5 \times 10^6$ Hz, $G = 2.947 \times 10^{10}$ Pa, $\rho = 2648$ kg/m³ and a $\Delta f_{\text{QCM}} = -45$ Hz, Eq. (2) gave $\Gamma = 7.9 \times 10^{-7}$ g/cm². The mass detection sensitivity of the PZT/gold-coated glass cantilever, $(\Delta m / \Delta f)_{\text{cant}}$ was therefore

$$\left(\frac{\Delta m}{\Delta f} \right)_{\text{cant}} = \frac{\Gamma A_{\text{cant}}}{\Delta f_{\text{cant}}}, \quad (3)$$

where Δf_{cant} and A_{cant} were the resonance frequency shift and the area of detection of the PZT/gold-coated glass cantilever. The detection area, $A_{\text{cant}} = 2wx \cong 3.2$ mm² where w the cantilever width, x the dipping depth, the numerical factor 2 taking into account that the cantilever tip was gold coated on both sides. With $\Delta f_{\text{cant}} = -450$ Hz, we obtained $(\Delta m / \Delta f)_{\text{cant}} = 5 \times 10^{-11}$ g/Hz. In comparison, with $\Delta f_{\text{QCM}} = -45$ Hz and the detection area, $A_{\text{QCM}} = 1.3$ cm², the mass detection sensitivity of the 5 MHz QCM was $(\Delta m / \Delta f)_{\text{QCM}} = \Gamma A_{\text{QCM}} / \Delta f_{\text{QCM}} = 2.3 \times 10^{-8}$ g/Hz. Clearly, in terms of $\Delta m / \Delta f$, the present PZT/gold-coated glass cantilever was more than three orders of magnitude more sensitive. A *Salmonella t.* cell is about 1.5 μ m long and less than 1 μ m wide with a mass about 1×10^{-12} g on average (see scanning electron microscopy (SEM) micrographs of *Salmonella t.* cells shown in Figs. 7). Therefore, in terms of *Salmonella t.* detection, only 50 cells were needed to cause a PZT/gold-coated glass cantilever to have 1 Hz frequency shift whereas more than 2×10^4 cells were needed to cause 1 Hz frequency shift in a QCM.

It is interesting to note that the experimental mass sensitivity deduced from the detection experiments was more than two orders of magnitude better than those obtained by the theory as shown in Fig 5(b). Considering a cantilever as an effective simple harmonic oscillator, its resonance frequency can be shifted by either a change in mass or a change in effective spring constant. The theoretical calculation shown in Fig. 5(b) considered only the change in mass. The more-than-two-orders-of-magnitude enhancement of the $\Delta m / \Delta f$ over the mass loading effect alone indicated that change in the effective spring constant was the dominant factor for the present resonance frequency shifts. Similar two-orders-of-magnitude mass sensitivity enhancements over the mass loading effect were also observed in other PZT microcantilever sensors, 14–16 and the change in effective spring constant was attributed to the induced stress by the atomic, molecular, or cellular attachment to the sensor surface. 14–16, 29–30 Exactly how the induced stress affect a cantilever's resonance frequency is not within the scope of this paper. A closer examination of the induced stress effect on a cantilever's mass sensitivity will be provided in a future publication.

III.4. *Salmonella t.* detection and mass detection sensitivity determination by cell counting

The antibody-coated cantilevers were then used to detect *Salmonella t.* at various concentrations for up to 30 min. In Fig. 8, we plot the resultant Δf versus time from the PZT/gold-coated cantilevers at various *Salmonella t.* concentrations. As can be seen Δf decreases sharply initially and reached a plateau after 10 min. The detections were repeatable as each curve was the average of 3–4 independent measurements. The standard deviations of Δf were about 10–20%. Table I lists the average values of Δf at $t = 30$ min for various *Salmonella* concentrations as obtained from the PZT/gold coated glass cantilever. As can be seen, at 5×10^3 cells/ml, the Δf obtained by the PZT/gold coated glass cantilevers at $t = 30$ min was -90 Hz well above the standard deviation 20 Hz and at 1×10^3 cells/ml, the obtained Δf was no longer meaningful compared to the standard deviation. This puts the detection limit of the current PZT/gold-coated glass cantilever at about 5×10^3 cells/ml. Note that since we did not control the humidity during the detection, to be conservative, we chose not to consider the background frequency up-shift. Since the possible background resonance

frequency up-shift could be as higher as +2 Hz/min or +60 Hz in 30 min, which had an opposite sign to the negative frequency shift due to detection. Therefore, if the background up-shift was taken into account, after correction, the magnitude of the negative frequency shift due to the cell detection could be even larger.

To validate the results shown in Fig. 8 and Table I, the PZT/gold-coated glass cantilever surface was examined by scanning electron microscopy (SEM) after 30 min of detection. Figures 9(a), 9(b) and 9(c) show the SEM micrographs of the captured *Salmonella* cells on the PZT/gold-coated glass cantilever surface at $t = 30$ min from 1×10^9 , 1×10^7 , and 1×10^5 cells/ml suspensions, respectively. With the mass of each cell being 1×10^{-12} g and by counting the number of cells in the micrographs, we obtained mass sensitivity $\Delta m/\Delta f$ of $-4 \pm 3 \times 10^{-11}$ g/Hz as estimated from the 1×10^9 , 1×10^7 , and 1×10^5 cells/ml suspensions, which was consistent with the value 5×10^{-11} g/Hz estimated from the antibody immobilization shown in Sec. III. 3. The error bar reflected the fluctuation in the cellular distribution on the micrographs.

III. 5. Comparison of *Salmonella t.* detection with and without humidity control

To ensure the above detection scheme by placing the water level at the cantilever's nodal point to afford detection without humidity control was indeed viable, we compared the detection of *Salmonella t.* without humidity control with detection of *Salmonella t.* at the same concentrations but with humidity control at 90% relative humidity using cantilever B. In the latter case, the water level of the cantilever was also at the nodal point. The only difference was that the relative humidity was held at 90% to ensure that there was no actual water level change during the course of detection and thus no background up shift. As an example, we plot in Fig. 10 the resonance frequency shift versus time for *Salmonella t.* detection at 10^5 cells/ml (open and full circles) and at 10^7 cells/ml (open and full squares) with and without humidity control with full symbols denoting 90% relative humidity and open symbols no control of humidity, respectively. As similar to all other results of this study, the data sets in Fig. 10 were the average of three independent detection tests. As can be seen, the curves of Δf versus time for detection tests at 90% relative humidity (full symbols) were essentially the same as those obtained without humidity control (open symbols) at both 10^5 and 10^7 cells/ml, validating the current nodal point approach. Note that the cantilever used was cantilever B which was different from cantilever A used for Fig. 8. Because the two cantilevers had somewhat different detection sensitivities, they exhibited different kinetic responses (cantilever A had a somewhat slower initial resonance frequency decrease than cantilever B). When the detection was done with the same cantilever, the kinetic response at the same concentration was repeatable with no more than 20% standard deviation.

III. 6. Comparison with QCM: *Salmonella t.* detection

For comparison, similar salmonella detection experiments were also carried out with the 5 MHz QCM without flow. The resultant average Δf versus time is plotted in Fig. 11. The values of the Δf obtained by the QCM at 30 min are also listed in Table I. As can be seen, the values of the Δf obtained from the 5 M Hz QCM were generally one order magnitude smaller than those obtained from the PZT/gold-coated glass cantilever and the standard deviations were about 20–30%, higher than those of Δf obtained by the PZT/gold-coated glass cantilevers. Moreover, at 1×10^5 cells/ml, the Δf obtained by the QCM was no longer meaningful when compared to the standard deviation and thus put the detection limit of the QCM at 1×10^5 cells/ml, which was about two orders of magnitude less sensitive than the 5×10^3 cells/ml detection limit of the PZT/gold-coated glass cantilever shown in Sec. III.4.

In summary, we plot the Δf at 30 min versus *Salmonella t.* concentration as obtained by the PZT/gold-coated glass cantilever (full circles) and the QCM (open triangles) in Fig. 12. Also shown in Fig. 12 were the infectious dose (open circle) and the detection limit of a commercial Raptor (open square) which also detects without labeling. The current 5×10^3 cells/ml detection limit was well below the infectious dose, 1×10^5 cells/ml,³¹ It was also lower than the 2×10^4 cells/ml detection limit of a commercial Raptor sensor.³²

IV. Conclusion

We have examined non-insulated PZT/gold-coated glass cantilevers for real-time, label-free detection of *Salmonella t.* by partial dipping at any relative humidity. The PZT/gold-coated glass cantilevers consisted of a 0.127 mm thick PZT layer about 0.8 mm long, 2 mm wide bonded to a 0.15 mm thick gold-coated glass layer with a 3.0 mm long gold-coated glass tip for detection. We showed that by placing the water level at the nodal point, which was about 0.8 mm from the free end of the gold-glass tip, we were able to have a 1/2 hr window in which the resonance frequency was stable in spite of the water level change by evaporation at all ambient humidity levels. We further showed that with the water level placed at the nodal point, the detection signal, Δf versus time was virtually independent of the relative humidity. This allowed the PZT/gold-coated glass cantilevers for real-time, label-free bio-detection without the need to insulate the PZT or control the humidity to minimize water evaporation. With a 0.8-mm dipping depth the background resonance frequency up shift was less than 2 Hz/min for any relative humidity $\geq 20\%$, and the mass detection sensitivity of the PZT/gold-coated glass cantilever was consistently determined as $\Delta m/\Delta f = -5 \times 10^{-11} \text{ g/Hz}$ from (1) the antibody immobilization detection result using the mass change per unit area obtained from the QCM and (2) from the *Salmonella t.* detection result by counting the number of cells captured on the cantilever surface. The PZT/gold-coated glass cantilever exhibited a detection limit of about 5×10^3 cells/ml, which was about two orders of magnitude lower than that of a 5 MHz QCM. It was about two orders of magnitude lower than the infectious dose and one order of magnitude lower than the detection limit of a commercial Raptor sensor. In addition, the present PZT/gold-coated cantilevers can be readily fabricated using commercial PZT and thus are convenient, practical and cost-effective.

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References

1. Brenner KP, Ranking CC, Roybal YR, Stelma GN Jr, Scarpino PV, Dufour AP. New Medium for the Simultaneous Detection of Total Coliforms and *Escherichia coli* in Water. *Appl Environ Microbiol.* 1993; 59(11):3534–3544. [PubMed: 8285660]
2. EPA. Microbiological Methods/On-line publication. URL <http://www.epa.gov/nerlcwww/online.htm>
3. Nie S, Zare RN. Optical detection of single molecules. *Annu Rev Biophys Biomol Struct.* 1997; 26:567–596. [PubMed: 9241430]
4. Chuang H, Macuch P, Tabacco MB. Optical Sensors for Detection of Bacteria. 1. General Concepts and Initial Development. *Anal Chem.* 2001; 73:462–466. [PubMed: 11217747]
5. Call DR, Brockman FJ, Chandler DP. Detecting and genotyping *Escherichia coli* O157:H7 using multiplexed PCR and nucleic acid microarrays. *Int J Food Microbiol.* 2001; 67(1–2):71–80. [PubMed: 11482571]

6. Fritz J, Baller MK, Lang HP, Rothuizen H, Vettiger P, Meyer E, Guntherodt H-J, Gerber Ch, Gimzewski JK. Translating Biomolecular Recognition into Nanomechanics. *Science*. 2000; 288(5464):316–318. [PubMed: 10764640]
7. Schemmel A, Gaub HE. Single molecule force spectrometer with magnetic force control and inductive detection. *Rev Sci Instrum*. 1999; 70(2):1313–1317.
8. Baselt DR, Lee GU, Colton RJ. Biosensor based on force microscope technology. *J Vac Sci Technol B*. 1996; 14(2):789–793.
9. G. U. Lee, D. A. Kidwell, and R. J. Colton, *U. S. Pat. Appl.* (1996) 38 pp. Avail. NTIS Order No. PAT-APPL-8-505 547.
10. Han W, Lindsay SM, Jing T. A magnetically driven oscillating probe microscope for operation in liquids. *Appl Phys Lett*. 1996; 69(26):4111–4113.
11. Thundat T, Wachter EA, Sharp SL, Warmack RJ. Detection of mercury vapor using resonating microcantilevers. *Appl Phys Lett*. 1995; 66(13):1695–1697.
12. Ilic B, Czaplewski D, Craighead HG, Neuzil P, Campagnolo C, Batt C. Mechanical resonant immunospecific biological detector. *Appl Phys Lett*. 2000; 77(3):450–452.
13. Oden PI, Chen GY, Steele RA, Warmack RJ, Thundat T. Viscous drag measurements utilizing microfabricated cantilevers. *Appl Phys Lett*. 1996; 68(26):3814–3816.
14. Lee JH, Kim TS, Yoon KH. Effect of mass and stress on resonant frequency shift of functionalized Pb(Zr_{0.52}Ti_{0.48})O₃ thin film microcantilever for the detection of C-reactive protein. *Appl Phys Lett*. 2004; 84(16):3187–3189.
15. Lee JH, Yoon KH, Hwang KS, Park J, Ahn S, Kim TS. Label free novel electrical detection using micromachined PZT monolithic thin film cantilever for the detection of C-reactive protein. *Biosensors and Bioelectronics*. 2004; 20:269–275. [PubMed: 15308231]
16. Lee JH, Hwang KS, Park J, Yoon KH, Yoon DS, Kim TS. Immunoassay of prostate-specific antigen (PSA) using resonant frequency shift of piezoelectric nanomechanical microcantilever. *Biosens Bioelectron*. 2005; 20(10):2157–2162. [PubMed: 15741091]
17. Hwang KS, Lee JH, Park J, Yoon DS, Park JH, Kim TSJ. In-situ quantitative analysis of a prostate-specific antigen (PSA) using a nanomechanical PZT cantilever. *Lab Chip*. 2004; 4:547–552. [PubMed: 15570363]
18. Shih WY, Li X, Gu H, Shih WH, Aksay IA. Simultaneous liquid viscosity and density determination with piezoelectric unimorph cantilevers. *J Appl Phys*. 2001; 89(2):1497–1505.
19. Yi JW, Shih WY, Mutharasan R, Shih WH. In situ cell detection using piezoelectric lead zirconate titanate-stainless steel cantilevers. *J Appl Phys*. 2003; 93(1):619–625.
20. Yi JW, Shih WY, Shih W-H. Effect of length, width, and mode on the mass detection sensitivity of piezoelectric unimorph cantilevers. *J Appl Phys*. 2002; 91(3):1680–1686.
21. Shen Z, Shih WY, Shih W-H. Mass detection sensitivity of piezoelectric cantilevers with a nonpiezoelectric extension. *Rev Sci Instrum*. 2006; 77:065101.
22. Campbell GA, Mutharasan R. Sensing of liquid level at micron resolution using self-excited millimeter-sized PZT-cantilever. *Sens. and Actuators A- Physical*. 2005; 122(2):326–334.
23. Campbell GA, Mutharasan R. Detection of pathogen *Escherichia coli* O157:H7 using self-excited PZT-glass microcantilevers. *Biosens Bioelectron*. 2005; 21:462–473. [PubMed: 16076436]
24. Zhu Q, Shih WY, Shih W-H. In-Situ, In-Liquid, All-Electrical Detection of *Salmonella typhimurium* Using Lead Titanate Zirconate/Gold-Coated Glass Cantilevers at any Dipping Depth. *Biosens Bioelectron*. 2007; 22:3132–3138. [PubMed: 17387007]
25. Langmuir I. Chemical reactions at very low pressures. I. The clean-up of oxygen in a tungsten lamp. *J Am Chem Soc*. 1913; 35(2):105–127.
26. Fung YS, Wong YY. Self-Assembled Monolayers as the Coating in a Quartz Piezoelectric Crystal Immunosensor To Detect *Salmonella* in Aqueous Solution. *Anal Chem*. 2001; 73:5302–5309. [PubMed: 11721933]
27. Hermanson, GT. *Bioconjugate Techniques*. Academic Press; 1996.
28. Sauerbrey G. Use of vibrating quartz for thin film weighing and microweighing (in German). *Z Phys*. 1959; 155:206–222.

29. Chen GY, Thundat T, Wachter EA, Warmack RJ. Adsorption-induced surface stress and its effects on resonance frequency of microcantilevers. *J Appl Phys.* 1995; 77(8):3618–3622.
30. Cherian S, Thundat T. Determination of adsorption-induced variation in the spring constant of a microcantilever. *Appl Phys Lett.* 2002; 80(12):2219–2221.
31. Pathirana ST, Barbaree J, Chin BA, Hartell MG, Neely WC, Vodyanoy V. Rapid and sensitive biosensor for Salmonella, Boisens. *Bioelectron.* 2000; 15:135–141.
32. Raptor Portable, Multianalyte Bioassay System. URL <http://www.resrchintl.com/raptor-detection-system.html>

Fig. 1.
(a) An optical micrograph and (b) a schematic of a PZT/gold-coated glass cantilever.

Fig. 2.

(a) Phase angle versus frequency resonance spectra in air (dashed line) and in water with a dipping depth of 0.8 mm (solid line) and (b) a schematic of the first- and second-mode flexural vibration wave forms of the PZT/gold-coated glass cantilever.

Fig. 3.

(a) A schematic of the PZT/gold-coated glass cantilever partially immersed in water with a dipping depth, x and (b) resonance frequency versus dipping depth, x .

Fig. 4.

Resonance frequency shift versus time of the PZT/gold-coated glass cantilever at various dipping depth, x in PBS at 20% relative humidity. Note that the resonance frequency shifts were positive. The rate of resonance frequency shift was represented by the slope of the curve, which exhibited a minimum at the nodal point, $x = 0.8\text{ mm}$.

Fig. 5.

(a) $\Delta f/\Delta t$ versus x at 20, 50, and 90% relative humidity, (b) $-\Delta f/\Delta m(x)$ versus x , and (c) $(\Delta f/\Delta t)/(-\Delta f/\Delta m(x))$ versus x of the PZT/gold coated glass cantilever where $\Delta f/\Delta t$ denotes the rate of resonance frequency change and, $-\Delta f/\Delta m(x)$ the theoretical resonance frequency shift per local unit mass decrease at x calculated using the theory of Ref. 21. Note the ratio, $(\Delta f/\Delta t)/(-\Delta f/\Delta m(x))$ gave the evaporation rate which was proportional to (1-relative humidity).

Fig. 6. Resonance frequency shift versus time of the PZT/gold-coated glass cantilever (full circles) and 5 MHz QCM (open squares) during (a) NHS and EDC modification, and (b) Antibody immobilization. The EDC and NHS modification and the antibody immobilization yield -90 Hz and -450 Hz shift after 30 min. In comparison, the QCM yielded no significant resonance frequency shift during EDC and NHS modification and -45 Hz shift for antibody immobilization.

Fig. 7.
A SEM micrograph shows the close-up of Salmonella t. cells.

Fig. 8.
Resonance frequency shift versus time of the PZT/gold-coated glass cantilever in various *Salmonella t.* concentrations.

Fig. 9. SEM micrographs the PZT/gold-coated cantilever surface after 30 min detection in a (a) 1×10^9 , (b) 1×10^7 , and (c) 1×10^5 cells/ml *Salmonella t.* suspensions.

Fig. 10.

Δf versus time of *Salmonella t.* detection with the water level placed at the nodal point with no humidity control (open circles and squares) and at 90% relative humidity (full circles and squares) at 10^5 cells/ml (circles) and at 10^7 cells/ml (squares). Each data set was the average of three independent detection tests. As can be seen, at the nodal point, the detection signal, Δf versus time was independent of the relative humidity level.

Fig. 11.
Resonance frequency shift versus time of the 5 MHz QCM in various *Salmonella t.* concentrations.

Fig. 12.

Resonance frequency shift at 30 min versus *Salmonella t.* concentration of the PZT/gold-coated glass cantilever (full circles) and that of the QCM (open triangles). Also shown were the infectious dose (open circles) and the detection limit of a commercial Raptor (open squares).

Table I

Resonance frequency shift at 30 min obtained by PZT/gold-coated glass cantilever and 5 MHz QCM

Concentration (cells/ml)	$-\Delta f$ (Hz) of cantilever	$-\Delta f$ (Hz) of 5 MHz QCM
1×10^9	970 ± 110	75 ± 10
1×10^7	590 ± 100	35 ± 10
1×10^5	300 ± 90	20 ± 20
1×10^4	160 ± 20	Not measurable
5×10^3	90 ± 10	Not measurable