Detection of Group A Streptococcus and Model Protein Using Self-Excited PZT-Glass Microcantilever

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ABSTRACT

A composite self-excited PZT-glass cantilever (4mm in length and 2mm width) was fabricated and used to measure attachment of model protein and a model pathogen. Key feature of the microcantilever is that its resonant frequency is dependent on its mass. The cantilever had a mass sensitivity of $5x10^{-11}$ g/Hz. The change in resonant frequency was measured as antibody, protein, reacts with the amine-derivatized sensing glass surface and pathogen, Group A Streptococcus, attaches to the antibody immobilized microcantilever. Very low pathogen concentration (640 bacteria/mL) was successfully detected, thus demonstrating the usefulness of the cantilever for detecting water-borne pathogens.

KEY WORDS: Resonant frequency change

INTRODUCTION

Resonant microelectromechanical systems (MEMS) have gained considerable interest over the last decade as sensors. Resonant MEMS rely on changes in its fundamental or higher mode resonant frequency changes to measure a number of different parameters, including absorption of biologicals [1-3], gases [4-7], chemicals [8-12] and others. More recently, applications in biological systems such as avidin-biotin [13,14], antibody-antigen interaction [15,16], and hybridization of complementary DNA strands [17] have been reported. Our own work relating to cellular attachment was based on non-specific binding of yeast on polylysine-coated stainless steel microcantilever with lead zirconate titanate (PZT) actuation [3]. In general, detection of a biological molecule or an entire bacterium requires the immobilization of a recognition molecule, such as an antibody (protein), on the sensor surface. When the target of interest binds to the cantilever's sensing surface, the effective mass of the cantilever increases which alters the cantilever's resonant frequency. Monitoring the resonant frequency change with time provides quantitative measures of the analyte. In this paper, we explore the application of PZT-glass microcantilevers for protein and pathogen detection. PZT-glass unimorph transducer is a flexural composite transducer with a PZT layer bonded to a non-piezoelectric one, glass in the present study. Applying an alternating current (ac) electric field in the thickness direction of the PZT layer sets the unimorph transducer into flexural oscillations by the converse piezoelectric effect. When the oscillation frequency is in the region of natural frequency of the cantilever, resonance is reached. At resonance, the resulting large flexural oscillations are detected electrically via the direct piezoelectric phenomena. At the last AIChE sensor symposium [18], we showed that by monitoring the resonant frequency change, a piezoelectric PZT-glass microcantilever could be used as a mass change detector. The mass change sensitivity, σ, is defined as the mass change that causes a 1 Hz frequency change. We reported earlier that σ values in the range of 10^{-10} g/Hz for a cantilever of length and width a few millimeter was successful in detecting pathogen E.coli 0157:H7.

In this paper, we report on the detection of model pathogen, Group A Streptococcus (GAS) and immunoaffinity purified monoclonal antibody (MAb) to GAS, at low concentrations of 640 bacteria/mL and 0.1mg/mL respectively, using PZT-glass microcantilever. The amine-derivatized glass cantilever tip was immobilized with MAb and subsequently the tip was exposed to various concentrations of the pathogen, GAS.

BACKGROUND

The piezoelectric PZT films give sensitive response to weak stresses due to the direct piezoelectric effect, and generate high strain via the inverse piezoelectric phenomena. The direct piezoelectric effect is used to excite the cantilever, and the same PZT film can be used to sense the resulting response. In general, a beam with a flexural rigidity of EI, where E is the modulus of elasticity and I is the moment of inertia, the natural frequency can be obtained by solving the general equation representing transverse mechanical vibrations:

$$EI\frac{\partial^4 y}{\partial x^4}+(\rho wt)\frac{\partial^2 y}{\partial \tau^2}+(c_0)\frac{\partial y}{\partial \tau}=0 \qquad (1)$$

where y is the displacement parallel to the thickness of the cantilever, x is length along the cantilever, τ is the time, and ρ is density. The term, c_0, is the damping parameter intrinsic to the cantilever. The moment of inertia, I, of a rectangular cross section is $wt^3/12$, where w is the width and t is the thickness. A number of previous investigators have determined solutions to the above equation [2,7,19,20]. A practical expression for the resonant frequency for sensing purpose is obtained when one considers the mass of cantilever to be located at the tip, and is given as [19,24]:

$$f_n=\frac{\nu_n^2}{2\pi}\sqrt{\frac{K}{M_e}} \qquad (2)$$

where ν_n is the eigen value corresponding to various oscillating modes. K and M_e are the effective spring constant and the effective mass of the cantilever, respectively. The parameter K depends on the thickness, density and Young's modulus of the cantilever material, namely both glass and PZT.

In the current application of pathogen detection in a fluid sample, added mass consists of two terms. First is due to the fluid surrounding the cantilever and the second is due to the attachment of the pathogen at the cantilever tip. Thus Eq (2) can be restated as:

$$f_{nf}^{'}=\frac{\nu_n^2}{2\pi}\sqrt{\frac{K}{M_{ef}+\Delta m}} \qquad (3)$$

where M_{ef} is the effective mass of the cantilever in fluid and $f_{nf}^{'}$ is the resonant frequency of the nth mode in fluid when pathogen of mass , Δm, is attached at the cantilever tip. Differentiating the above equation gives

$$f_{nf}^{'}-f_{nf}=\frac{1}{2}f_{nf}\frac{\Delta m}{M_e} \qquad (4)$$

where f_{nf} is the resonant frequency of the nth mode in fluid. That is, a change in resonant frequency (represented by the left hand side of Eq (4) is linearly dependent on the change in mass, at a particular resonant mode. The other parameters in Eq (4) are constants for a given cantilever. The effective mass in fluid, M_{ef}, can be determined experimentally from the resonant frequency under liquid immersion conditions. Thus, with revised values of cantilever mass one can use Eq (4) to calculate mass change of cantilever due to added mass. In this paper, we will use the above to estimate GAS attachment to antibody-immobilized cantilever.

MATERIALS AND METHODS

Microcantilever Fabrication

Construction features of the PZT-glass microcantilever are shown schematically in Figure 1. It was fabricated using two main components. 127 μm thick PZT single sheet (Piezo Systems Inc., Cambridge, MA), and 160μm thick cover glass slip (Fisher Scientific).

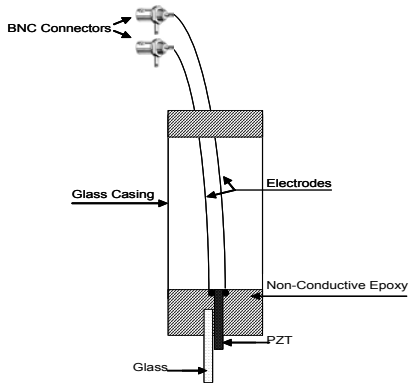

Figure 1. A schematic of PZT-Glass microcantilever geometry.

The PZT and silica glass cover slip were cut to 2mm x 5mm and 2mm x 4mm, respectively, using a diamond knife. The PZT layer was bonded to the glass with a non-conductive epoxy (Loctite) and then was clamped between two glass slides for at least 2 hours for curing to take place at room temperature. After this, the sides of the PZT layer were sanded so as to ensure alignment with the glass layer. The top nickel surface of the PZT, which serves as the top electrode, and the bottom nickel surface, serves as the bottom electrode, were connectarized using 50 gauge copper wire soldered to BNC couplers. The electrode end of the cantilever was encapsulated in a glass tube by a non-conductive epoxy (VS-101, Huntington Mechanical Laboratories Inc., CA).

In Figure 1, it can be seen that the glass layer at the cantilever tip is longer than the PZT layer to allow for attachment of sensing molecule, antibody.

Experimental Arrangement

The fabricated microcantilever was firmly attached to a XYZ position manipulator (Sigma Corp., Santa Ana, CA) using a clamp. The manipulator was used to adjust the cantilever in vertical direction from its reference point to various immersion depths in the sample containing beaker. Typical sample volume was one mL. The cantilever electrodes were connected to an impedance analyzer (Agilent, HP4192A) interfaced to a PC for obtaining impedance, phase angle and amplitude ratios at various frequencies in the range of 1 to 100 kHz with an excitation voltage of 100 mV.

Antibody Immobilization

The sensing glass surface was thoroughly cleaned sequentially with methanol-hydrochloric acid solution (1:1 v/v), concentrated sulfuric acid, hot sodium hydroxide, and finally boiling water [21]. The surface was rinsed between each washing steps with deionized water. The cleaning procedure produces reactive hydroxyl groups on the glass surface. After cleaning, the glass surface was silanylated with 10% 3-aminopropyl-triethoxysilane (APTES; Sigma-Aldrich) in deionized water at pH 3.0 (adjusted by hydrochloric acid, 10 N) and 75^{o}C for 2 hours. APTES reacts with glass leaving a free amine terminal for further reaction with carboxylic group to form a peptide bond. The carboxyl group present in the monoclonal antibody, MAb, (ViroStat) to group A streptococcus (Immuno Resources Inc.) was activated using the zero length cross linker 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; Sigma-Aldrich) and promoted by sulfo-N-hydroxysuccinimide (Sigma-Aldrich). EDC converts carboxylic groups into reactive unstable intermediates susceptible to hydrolysis. Sulfo-NHS replaces the EDC producing a more stable reactive intermediate that is susceptible to attack by amines. Covalent coupling of the stable intermediate with the silanylated glass surface was carried out at room temperature for 2 hours. The glass surface with the immobilized antibody was used to detect the pathogen group A streptococcus. The immobilization protocol was taken from Bioconjugate Techniques [22] and modified as per sample size.

Experimental Determination of Mass change Sensitivity

Point masses of silicone oil (50 cp) were dispensed at the cantilever tip using a tapered 670 microns silica fiber. The tapering was done by flame drawing using a micro-torch (Microflame Inc., Plymouth, MN). The tapered end of the fiber, 20 microns in diameter, was dipped in the silicone oil to a prescribed depth, followed immediately by touching the cantilever surface. This was executed by the same person, and with due practice reasonable reproducibility was achieved. Error in measuring mass of 0.02 mg was $\pm$0.0033mg. The resonant frequency of silicone-added cantilever was measured in air. The procedure was repeated for six mass changes, and several times for each mass change. Eq (4) was used to determine the added mass of silicone oil. The resonant frequency changes resulting from these specific mass changes were plotted to obtain experimental measures of mass sensitivity of the cantilever.

Detection Experiments

Group A streptococcus stock solution ($7x10^9$ bacteria/mL) was purchased and a low concentration, 640 bacteria/mL, of the antigen was prepared in PBS by serial dilution. Antibody to the antigen stock solution (1 mg/mL) was prepared using vendors protocol in 10 mM phosphate buffered saline solution pH 7.4 (PBS). One mL of the antibody sample was loaded into a 1 mL container, and the silanylated sensing tip of the cantilever was immersed to a depth of 1mm using the XYZ-manipulator. The impedance and phase angle of the PZT-layer at 1 to 100 kHz was monitored and recorded. Subsequently, the cantilever was immersed to 1 mm in the antigen sample for one hour while monitoring the second resonant mode.

RESULTS AND DISCUSSION

Resonance in air

Resonance spectrum of the cantilever used in this study (physical dimensions in Table 1) in air is shown in Fig.2. The numerical results and key performance parameters are given in Table 2.

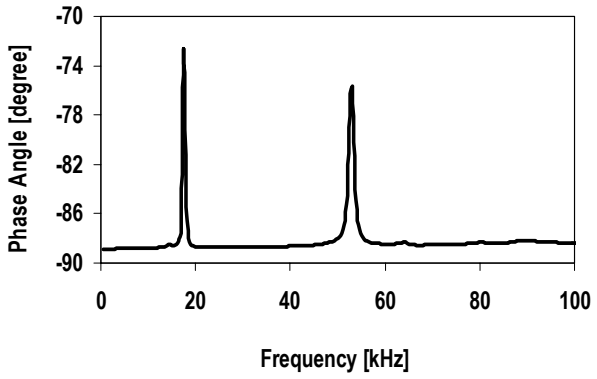

Figure 2. Resonant spectrum of phase angle versus frequency of the cantilever in air. At resonance, the phase angle of the oscillating cantilever (100mV excitation) exhibits a sharp peak.

The first peak represents the fundamental frequency, and the higher modes occur at higher frequencies. Note that only two resonant frequencies (17.5 and 53.5 kHz) were found in the frequency range, 1 to 100 kHz. It is also interesting to note that the ratio of second mode frequency to the first mode is 3.14. The relationship given for a uniform rectangular cross-section cantilever (Eq (2)) predicts a value of 6.27. The difference is primarily due to the non-uniform cross section and the two-layer construction.

Table 1. Cantilever physical dimensions

Dimensions	PZT	Glass
L (mm)	1	3
W (mm)	2	2
t (μm)	127	160

Table 2. Cantilever performance parameters

Mode	Resonant frequency [kHz], in air	Q factor in air	Resonant frequency [kHz], in PBS 1mm	Q factor in PBS 1mm
1	17.5	25	11.5	------
2	53.5	36	44.5	32

Quality Factor in Air

The mechanical quality factor (Q factor) characterizes the sharpness of the peak. The Q factor is defined as the ratio of the resonant peak frequency relative to the resonant peak width at half peak height. As shown in Table 2, typical values range from 30 to 100, and this value does not deteriorate significantly upon immersion in water-like fluid. While there is a loss of about 11% in Q value going from air to one millimeter immersion depth in PBS, the decrease is far less than what one observes with silicon cantilevers [25].

Mass Sensitivity

The mass change sensitivity was determined by the dip-touch silicone oil technique [26]. For the cantilever size investigated (in Table 1) the value of the mass sensitivity (σ) is 5×10^{-11} g/Hz. This suggests that if resonant frequency resolution is 1 Hz, then mass changes of 50 pg are discernable.

Resonant frequency in liquid immersion

In Fig. 3 the phase angle is plotted against frequency for the second flexural mode. The resonant frequency decreases from 53.5 kHz in air to 44.5 kHz in PBS.

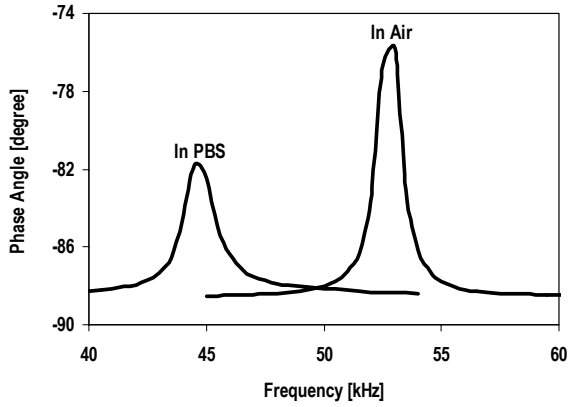

Figure 3. Second flexural mode resonance peaks of the cantilever in air (right) and in PBS solution (left) immersed to a depth of 1mm. The liquid adjacent to the cantilever detecting surface became part of its effective mass. As a result its resonant frequency decreased.

Resonant frequency is directly proportional to the inverse square root of the cantilevers mass, therefore, on increase in mass due to the liquid causes a decrease in its natural resonant frequency. The quality factor of the resonant peak decreases from 36 in air to 32 in PBS.

Reaction of MAb to amine-terminal silane cantilever surface

The cantilever response to samples containing the antibody to GAS at concentrations of 0.1 mg/mL and 1 mg/mL is shown in Fig. 4. Here the EDC/sullfo-NHS activated MAb reacts with the amine group on the glass surface to cause mass change of the cantilever. In essence, the response following the peptide bond formation is in real-time. For the higher concentration, the resonant frequency decreases more rapidly, and reaches a constant value. Total change is approximately 1600 Hz. At the lower concentration, 0.1 mg/mL, the change is far slower and steady state was achieved at a different value, 600 Hz, presumably corresponding to lower reaction equilibrium. Using the mass change sensitivity (σ) of the cantilever, $5x10^{-11}$ g/Hz, the mass of protein attached in the first 60 minutes from the highest to the lowest concentration are, respectively, 80 and 30 ng. The attachment surface area is 4.64 mm^2, and an average antibody has a projected area of 6.16×10^{-12} mm^2 and thus the maximum mass change that can be attained for complete monolayer coverage of the exposed surface is 190 ng. Although direct quantitative chemical assays were not performed, the results are reasonably consistent within experimental bounds.

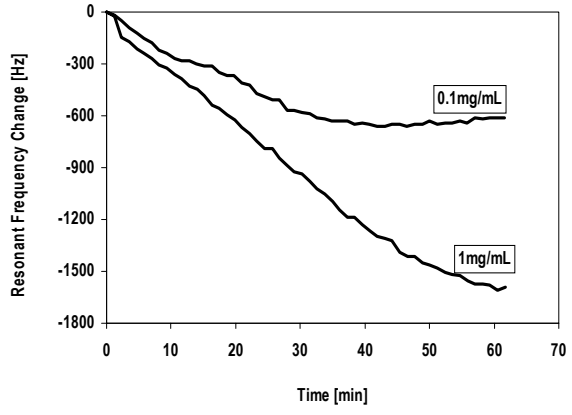

Figure 4. Resonant frequency change versus time of the cantilever, due to the reaction of MAb (sample concentrations 0.1mg/mL and 1mg/mL) and the amine-group of the silanylated glass surface.

Response to different concentrations of GAS

Fig. 6 shows the attachment of Group A Streptococcus of concentrations $7x10^9$ bacteria/mL and 640 bacteria/mL to the cantilever bearing antibody (MAb), 1.0 mg/mL.

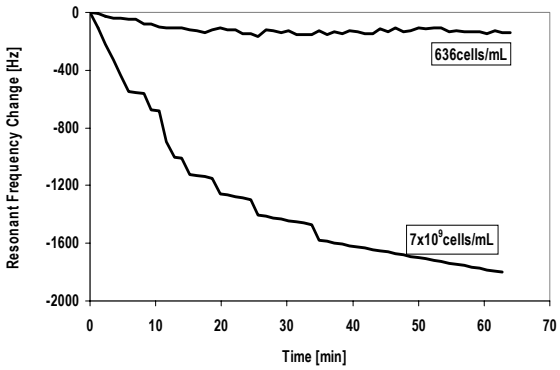

Figure 6. Resonant frequency change versus time of the second flexural resonant mode for the cantilever bearing MAb (1.0 mg/mL), in detecting the attachment of 640 bacteria/mL and $7x10^9$ bacteria/mL samples of GAS. The figure showed an increase rate of attachment as the bacteria concentration increases. The total change in resonant frequency also increases with bacteria concentration.

Clearly, the higher the bacteria concentration the greater the resonant frequency change and its rate of change. Here, it is noteworthy to emphasize that for the first 60 minutes of the experiment the sample containing $7x10^9$ bacteria/mL did not reached saturation. However, the low concentration sample, 640 bacteria/mL, reached a constant value (144 Hz) in the first 30 minutes of the attachment experiment. This corresponds to mass of pathogen attached as 90 and 7.2 ng from the $7x10^9$ bacteria/mL and 640 bacteria/mL samples, respectively. These results suggest that the PZT-glass microcantilever is able to detect very low concentrations of protein and bacteria with good sensitivity.

SUMMARY

In this paper we have shown that: (1) A PZT-glass cantilever suitable for pathogen detection was fabricated, and its frequency response characterized using reported vibration models for resonance. (2) Antibody reaction to an amine-derivatized glass surface was successfully monitored in real-time. (3) Successful detection of Group A Streptococcus at a concentration of 640 bacteria/mL was achieved.

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