Available online at www.sciencedirect.com
SCIENCE DIRECT®
Sensors and Actuators B 96 (2003) 565–575
SENSORS AND ACTUATORS B CHEMICAL
www.elsevier.com/locate/snb

Detection of proteins and intact microorganisms using microfabricated flexural plate silicon resonator arrays

Jane Pepper [a,*], Richard Noring [b], Mark Klempner [b], Brian Cunningham [a], Anthony Petrovich [a], Robert Bousquet [a], Christopher Clapp [a], Jeanne Brady [a], Brenda Hugh [a]

[a] *C.S. Draper Laboratory, 555 Technology Square, Cambridge, MA 02139, USA*
[b] *New England Medical Center, 750 Washington St., Boston, MA 02111, USA*

Received 3 January 2003; accepted 22 July 2003

Abstract

We are developing biosensor arrays that are based on microfabricated silicon flexural plate wave (FPW) resonators coated with molecular recognition chemistry. The resonators within the micro-chemical analysis array (μCANARY) are micro-electromechanical (MEM) sensors that have been miniaturized to allow many independently addressable sensors to be integrated within a single silicon chip. The target analyte of an individual sensor within the chip is selectively detected by depositing molecular recognition component (or "coating") onto the sensor surface, and monitoring changes in the frequency and phase of the resonance as the coating interacts with the analyte. The ultimate goal of this project is integration of hundreds of miniature resonators within a single chip for detection of biological species. As proof of concept demonstration, we describe here the detection of proteins and intact microorganisms using 2-element and 8-element μCANARY sensor chips and address electronics. Preliminary results of sensitivity, selectivity, and surface regeneration methods of the sensor are presented. Detection of proteins and microorganisms with the μCANARY sensor were confirmed by optical measurements.

Keywords: FPW sensor; MEMS; Biosensor; Sensor array; Protein; Microorganism

1. Introduction

With the completion of the sequencing of the human genome [1], one of the next grand challenges for molecular biology will be to understand the regulation and function of the proteins that can be expressed by cells. To this end, tools that have the ability to simultaneously detect the presence of many different proteins at low concentrations within a sample will find applications in pharmaceutical discovery, environmental testing, clinical research, and diagnostics. Further, for these tools to find widespread use, they must be simple to use, inexpensive to buy and operate, and applicable to a wide range of analytes, which may include small peptides, proteins, or even entire cells.

Detecting and identifying proteins and pathogens with high sensitivity, high selectivity, and high speed in a complex mixture is a challenging task. Detection sensitivity and speed are of particular importance for analytes that have no convenient means for rapidly amplifying concentration. Several commonly used approaches for measuring complex protein mixtures such as Western blotting, ELISA, gel electrophoresis, and HPLC, are amenable to small numbers of analytes and usually require extensive sample preparation and/or a waiting period before the results can be analyzed. Methods such as mass spectroscopy are powerful and sensitive, but require complex and expensive instrumentation.

Supported by the advances in micro-fabrication methods, sensor microarrays that provide a high degree of redundancy have become attractive to address the issue of detecting many analytes simultaneously [2–4]. Many of the currently available array methods, however, are based on fluorescence or particle labeling. Meanwhile, several direct optical sensing methods based on surface plasmon resonance (SPR) [5], ellipsometry [6], and optical waveguides [7] have been demonstrated. Of these methods, none are capable of measuring hundreds to thousands of biochemical processes simultaneously, and several require the use of laser excitation or an optical system that requires precise alignment. The approach that we describe here aims to overcome the challenges of sensitivity and speed of measuring complex

* Corresponding author. Present address: 77 Edgelawn Ave, N. Andover, MA 01845, USA.
E-mail address: jpepper100@yahoo.com (J. Pepper).

0925-4005/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0925-4005(03)00641-5

mixtures of proteins using multiplexed, simple circuitry, and label-free methods.

This paper describes a microfabricated silicon chip containing an array of miniature sensors that detect analytes by the mass that they deposit upon individual locations within the array. The micro-chemical analysis array (μCANARY) utilizes miniature flexural plate wave (FPW) silicon resonators coated with affinity ligand reagents (ALRs) deposited onto individual sensors with a microdroplet applicator. Because the size of an individual sensor element is very small, many sensors—each targeted at a different analyte—can be integrated within a single small silicon chip. This enables independent monitoring of many biochemical interactions simultaneously, and provides a detailed "fingerprint" of an analyte's properties. This increased analytical capability can be used to improve the ability of a system to rapidly screen a multitude of biochemical interactions. It can also be used to discriminate a particular analyte chemical in the presence of interfering substances (i.e. a type of non-specific binding), which reduces the rate of false alarm. The μCANARY operates in air or liquid environments, utilizes low-cost, low-power address/excitation/readout circuitry, and has demonstrated high detection sensitivity. The ultimate goal of this project is integration of hundreds of miniature resonators within a single chip.

With appropriate molecular recognition chemistry, the μCANARY is capable of detecting proteins and bacterial agents without the use of labeling reagents or signal amplification. The magnitude of the mass reduction is proportional to the number of protein molecules or bacterial agents bound to the receptor coating on the sensor surface, which in turn is an indication of the affinity of the ALR for the analyte. This approach is advantageous because the sensor chip can be inexpensively batch-fabricated by a silicon foundry using a simple 3-photomask process, while the readout electronics utilize only commercially available components. Further, once the chip is activated with microdroplets of ALRs using a high-throughput instrument that is typically used to produce DNA microarrays, the sensor excitation and readout is performed electronically, without the need for label reagents or an optical excitation/transduction system. Adsorption of mass onto the sensor membrane can be detected in real time. The detection system allows for simple incorporation of a reference resonator that can effectively remove common-mode effects such as temperature and non-specific adsorption. Finally, currently available sensors have the ability to resolve mass changes of approximately 2 pg (in air) [8]. Any type of analyte molecule or particle can be detected provided that enough of them can be captured by the receptor coating to register a signal above background resonant frequency variation.

This work builds upon the invention of the FPW sensor by Wenzel and White, and its subsequent use as a chemical sensor [9–11]. Zellers and coworkers have also developed an FPW sensor array for vapor detection [12]. Mathematical models for designing miniature FPW sensors, their application toward detection of chemical weapon vapors, and a fabrication process for 2-element μCANARY chips have been described in separate publications [8,13]. To use the μCANARY sensor for bio-detection, we have also taken advantage of the extensively studied self-assembled monolayer (SAM) sensor coating technology [14], which can be readily adapted to the gold surface of the μCANARY sensor. In this paper, we describe: (1) the fabrication, packaging, address electronics, and resonant mode tracking method for an 8-element array of μCANARY sensors, and (2) the development of surface chemistry that enables regenerable detection of both protein and microorganisms such as bacteria and spores, with the detection confirmed by optical measurements. These studies are a prelude to extending the size of the sensor array to much larger formats that will enable hundreds of analytes to be tested simultaneously.

2. Sensor design

The fabrication sequence for building the Draper FPW resonator (cross-section diagram of a completed device is shown in Fig. 1A begins with a purchased silicon-on-insulator (SOI) wafer (BCO Technologies, Belfast, UK). The SOI wafer upper surface is a 2 μm thick layer of epitaxial silicon bonded to a 1 μm thick layer of SiO_2. The SOI substrate is ∼380 μm thick. A layer (∼0.5 μm) of piezoelectric AlN is deposited over the upper epitaxial silicon [15,16].

Vias for grounding contacts to the epitaxial silicon are provided by etching an opening into the AlN. Next, TiPtAu metalization of 0.1 μm total thickness is patterned to define interdigital metal electrodes, wire bond pad areas, and ground contacts. Finally, the membrane is defined by etching a vertical sidewall cavity from the back side of the wafer with an inductively coupled plasma (ICP) etch machine using the Bosch process [17]. The 1 μm SiO_2 layer of the SOI substrate acts as an automatic etch stop for the ICP process. After the ICP etch is completed, the SiO_2 layer is removed by dipping the wafer into buffered hydrofluoric acid.

The layer thickness and metal electrode widths are selected to excite a membrane resonant mode of ∼25 MHz in air. The electrodes are positioned within the membrane to align with eigenmodes of the resonating plate in order to maximize the amplitude of a particular resonant mode. The detailed modeling and design of the miniature FPW resonator have been described previously [13]. SEM photos of a single miniature FPW sensor, and several sensors within an array are shown in Fig. 1B and C. As shown in Fig. 1B, two sets of metal electrodes are utilized: one pair of electrodes applies a drive signal to excite the resonance, while the second set of electrodes senses the output response. The drive and sense electrode structures are identical, and can be used interchangeably.

The electronics for the sensor are designed for open-loop operation through an interface with a network analyzer. The

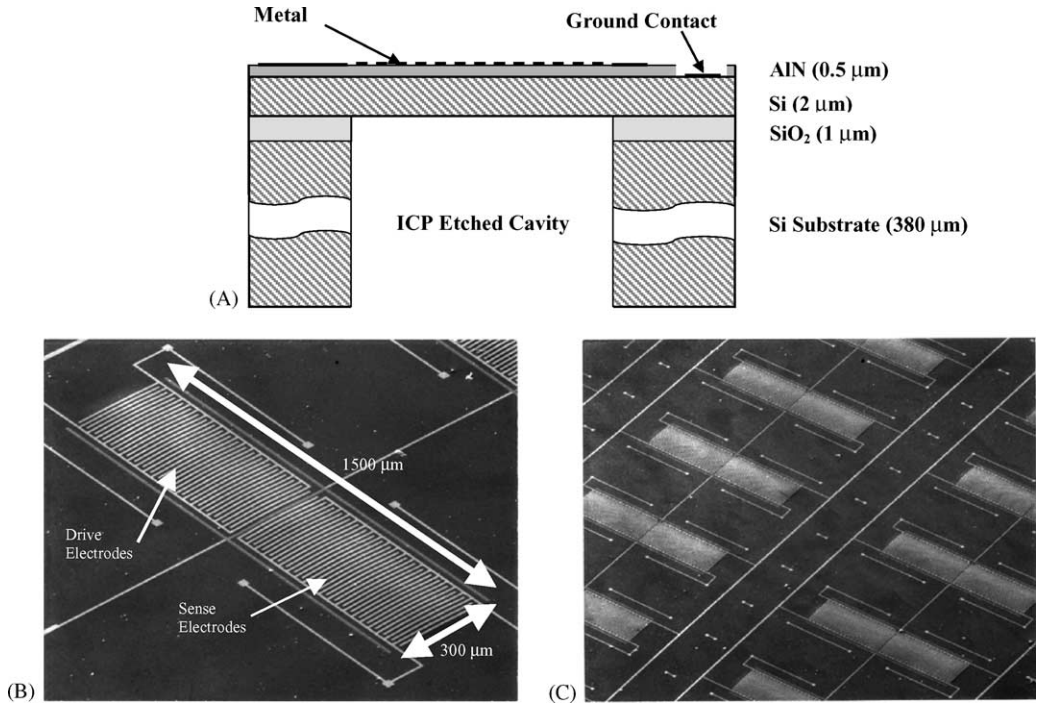


Fig. 1. (A) Cross-section diagram of the FPW resonating membrane structure, (B) SEM photo of a sensor element from the top surface of the wafer showing detail of metal electrode structure, and (C) SEM photo of several integrated FPW membranes within a section of an integrated sensor array.

electronics require a source input from the analyzer, and provide a reference signal (identical to sensor excitation) as well as the final gained output signal to the analyzer. The drive section consists of a high-gain single-ended input to differential output amplifier, which is capable of driving capacitive loads at high frequency. The drive select uses a data select circuit to enable unique pairs of active FET switches allowing for individual differential drive of each sensor in the array. The pre-amp uses high-gain amplifiers configured as an instrumentation amplifier. This configuration allows for symmetrical loading on the sensor output, high common-mode signal rejection, and higher gains for a given limited bandwidth.

The μCANARY measures the adsorption of selectively captured analytes directly (i.e. without labeling) through the mass that they deposit onto the silicon resonator. The resonance frequency of the sensor membrane decreases proportionally with the increase of this captured mass. The amplitude and phase of the resonance are monitored throughout the detection in real time. The electronic architecture required to operate the μCANARY resonator array is designed to minimize the size, cost, and power of the detection system. The sensors were developed first in 2-element then expanded to 8-element arrays. First generation sensors have demonstrated the speed and sensitivity (∼20 ppb) of the μCANARY in detecting vapor-phase chemical weapon simulants. [8] For second generation (i.e. 8-element) sensor arrays, a single sensor “drive” circuit is sequentially addressed to resonate each element of the sensor array through a multiplexer (Fig. 2). The resonator “sense” amplifier circuit is also shared among all elements of the array. In addition, a phase locked oscillator circuit is designed to accommodate a wide range of sensor characteristics and provide multi-function readout that includes resonant frequency, phase, attenuation, and Q. Using this arrangement, the entire readout circuit of a μCANARY array chip can be built using a small circuit board with only ∼7 commercially available integrated circuit chips, at a cost of ∼US$ 200. A laptop computer is currently used to access and process the signals generated by the readout circuit. However, it is anticipated that a miniature application-specific device approximately the size of a handheld calculator could be built to perform the analytical functions currently performed by computer. A multiple-element μCANARY is shown in Fig. 3.

To provide accurate compensation for common mode noise sources (e.g. temperature variation, humidity variation, and solution viscosity, etc.), a “reference” oscillator with close physical proximity and identical structure to “sample” sensor oscillators is incorporated. Such a sensor cross-correlation improves the stability of sensor frequency reading, an example of which is shown in Fig. 4 where two uncoated elements on a multi-sensor array were both exposed to air. The temperature of air dropped during the first approximately 30 min and then stabilized, which caused the readings on both sensors to drift upward at the beginning before stabilizing. As shown in Fig. 4C, using an oscillator as reference to compensate for common mode noise, the stability of sensor reading improves.

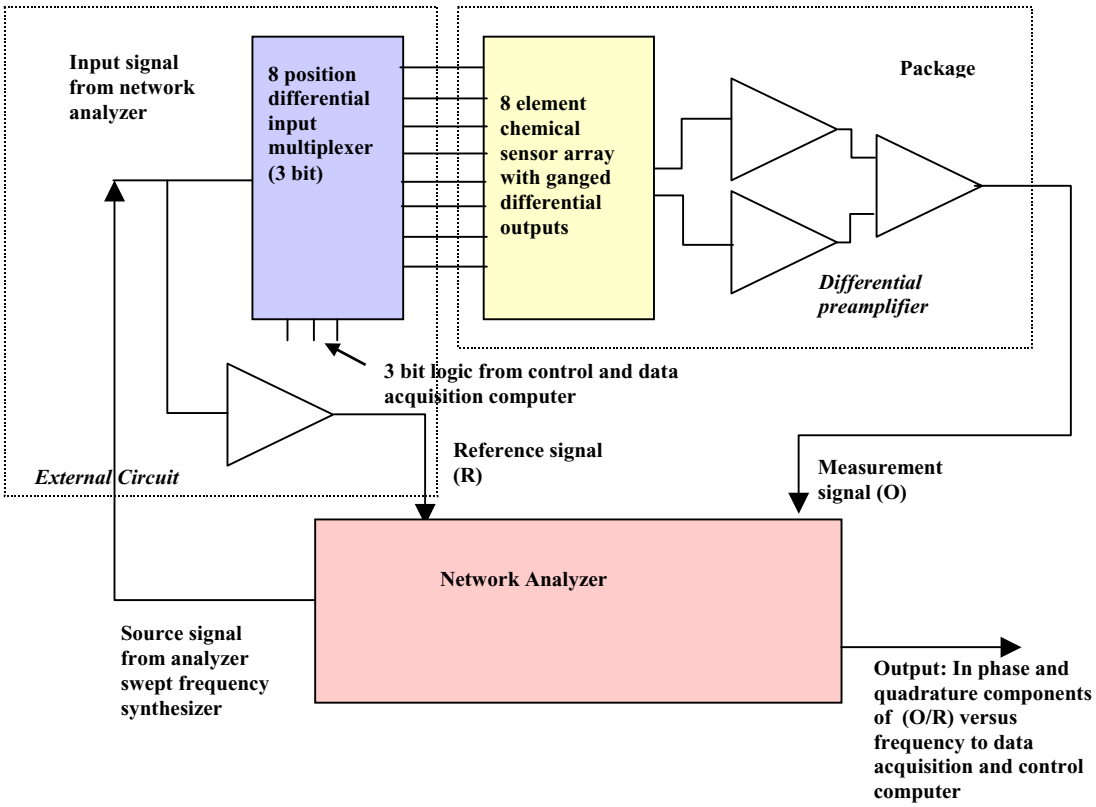


Fig. 2. Schematic of circuitry for 8-element μCANARY sensor in open-loop measurement.

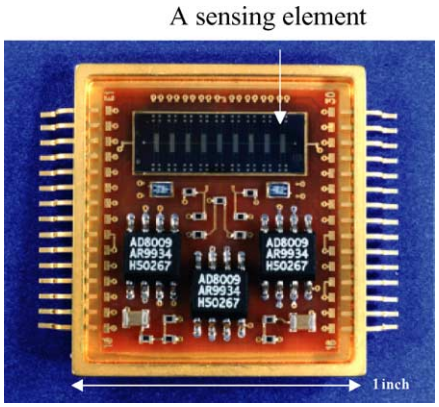


Fig. 3. Photo of a multiple-element μCANARY sensor package.

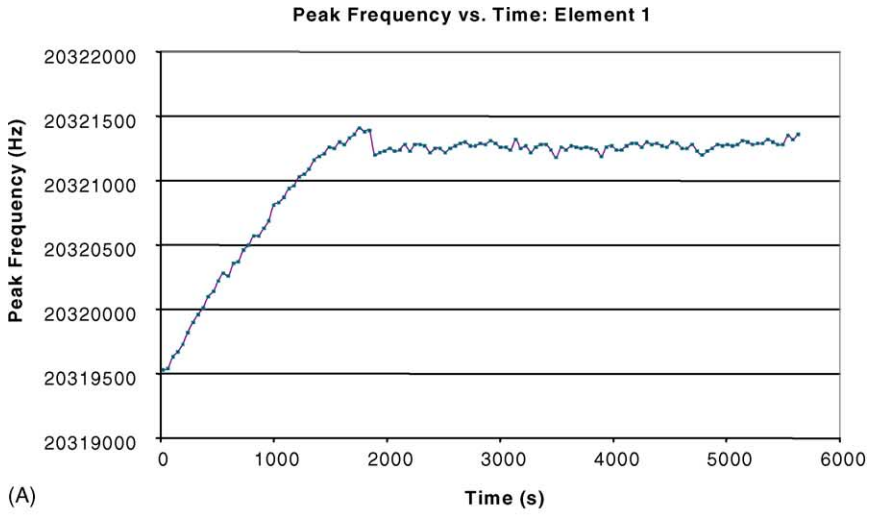
3. Experimental

To use the μCANARY sensor to detect biological species, a flow cell made of stainless steel was constructed to mount on the "back side" of the sensor package (i.e. the side without electronics). The flow cell was driven by a syringe pump and enabled liquid to contact the sensor surface.

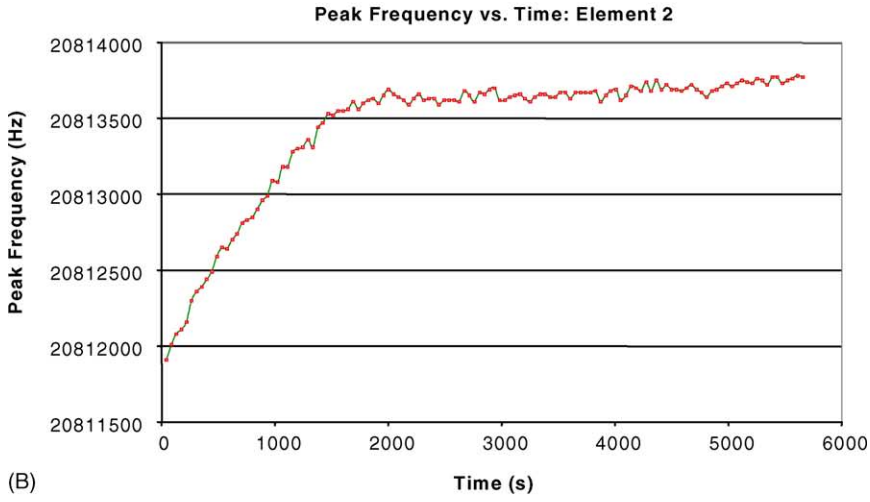
Reagents of 2-aminoethanethiol, glutaraldehyde, dog-IgG, anti-dog IgG–biotin conjugate, avidin, interleukins (ILs) and their antibodies (anti-ILs) were obtained commercially from either Sigma or R&D Research and used without further purification. Maltose, maltose-binding protein, amylose, bacteria *Borrelia burgdorferi*, outer surface protein A (OspA) of the bacteria and its antibodies (anti-OspA), and serum samples from vaccinated and non-vaccinated patients were all obtained at the New England Medical Center (NEMC).

The MEMS sensor fabrication process included sputtering a 400 Å layer of gold on the sensor surface to facilitate surface activation for the receptor binding process. For detection of dog-IgG (R&D Research) and interleukins (ILs, from R&D Research), the gold sensor surface was first activated by 2-aminoethanethiol (Sigma) to form a self-assembled monolayer on the surface through the strong bond of Au–S. The sensor was then sequentially exposed to glutaraldehyde (Sigma) and avidin (Sigma) to immobilize avidin on the surface. Biotinylated antibodies against the analytes, anti-dog IgG and anti-ILs (all from R&D) were then immobilized as receptor proteins to capture, selectively, the target species. For detection of anti-outer surface protein A (anti-OspA, obtained at NEMC), amylose was immobilized on the sensor to bind maltose-binding protein (MBP)–OspA complex (obtained at NEMC), which acted as a capture probe to detect anti-OspA. For detection of bacteria *B. burgdorferi*, biotinylated human IgG was used to capture human anti-OspA on the surface, which in turn bound to OspA on the bacteria.

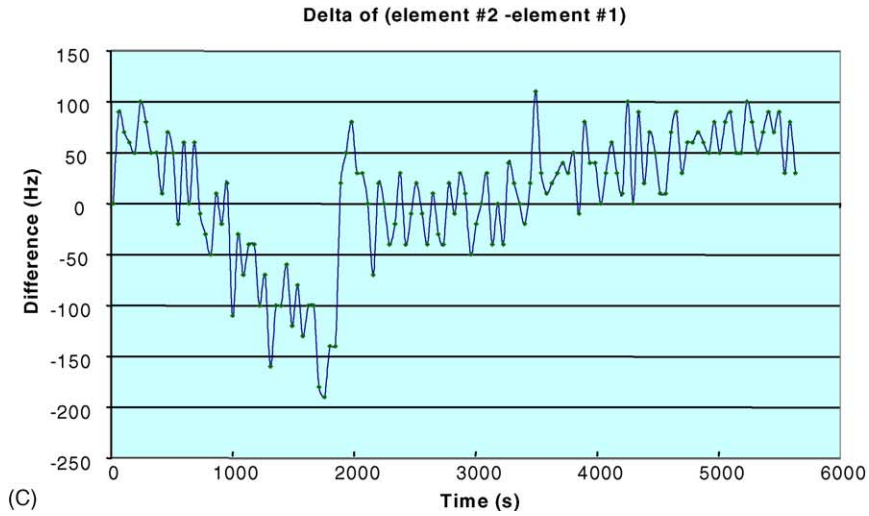
For blocking non-specific binding of proteins on the sensor, bovine serum albumin (BSA) solution was utilized. After each step of immobilization, the sensor surface was washed with PBS to remove unbound reagents.



(A)



(B)



(C)

Fig. 4. Resonance frequency readings in air of two elements on a multi-sensor array over a 100 min period. (A) Peak frequency reading for element 1; (B) peak frequency reading for element 2; (C) peak frequency reading difference between elements 2 and 1.

In order for each sensor in the array to independently recognize a different analyte protein, a BioDot^{TM} microdroplet applicator (Cartesian) was used to deposit receptor protein monolayers onto individual sensors. In the experiment where a multi-element sensor array was used to identify serum samples from patients who had been either vaccinated against Lyme disease or prescribed placebo, the applicator was used to deposit 50 nl of patient sample onto individual sensors.

To confirm the results obtained with μCANARY sensors, ellipsometry and confocal microscopy studies were also performed.

4. Results and discussions

4.1. Detection of dog-IgG

A large number of bioassays are based on the interaction of antibodies and antigen. One of our goals was to devise a "generic" method for preparing the sensor with a surface-immobilized antibody that targets the protein of interest. Ideally, the surface activation protocol will be identical for every sensor, except for the application of the antibody material as a final step. One strategy that has been used very successfully for assays is to use antibodies that had a biotin molecule attached to an inactive site of the antibody molecule. A surface coated with avidin has a very high affinity for the biotin, and binds the antibody in place, in the correct orientation for optimal interaction with the analyte. Many bioassays have taken advantage of the very strong avidin–biotin interaction. In addition, many sources provide biotinylation kits and biotinylated antibodies. As a result, we were able to purchase many antibodies for the proteins of interest to us. Therefore, the main goal of surface activation entails obtaining covalent attachment of an avidin monolayer to our MEMS resonator.

Early development of receptor surface chemistry involved attachment of receptor molecules to a bare silicon surface. This required silanization of the silicon surface—a chemical treatment process that involved baking the packaged sensor at an elevated temperature to obtain proper activation. The elevated temperature process was found to relax strain in the packaging materials, contaminate sensors with residues from the oven, and to result in unpredictable shifts in sensor resonant frequency.

The MEMS sensor fabrication process was modified to include a thin gold layer on the active surface to facilitate the receptor binding process. The gold surface is activated at room temperature by an aminoalkanethiol compound, which sequentially binds to glutaraldehyde, avidin, biotinylated antibody, and finally the specific antigen. To test the effectiveness of the procedure, preliminary studies were conducted on dummy wafers with gold sputtered on the surface. These wafers were first tested with coatings of biotinylated anti-dog IgG and dog-IgG. Ellipsometry measurements were used to confirm that the bio-layers had been coated on the gold surface. The results indicated that the total thickness for the first several coating layers with biotinylated anti-dog IgG was 144 ± 84 Å (the standard deviation of 10 readings across the chip reflected the uneven coating on the 0.5 in. × 0.5 in. surface, which is much larger than the 300 μm × 1500 μm sensor area). When dog-IgG was bound to the surface, the total thickness increased to 272 ± 145 Å. Negative control measurements were done by directly immersing the wafers in: (1) biotinylated anti-dog IgG, and (2) first in biotinylated anti-dog IgG and then in dog-IgG without covalently linking the anti-dog IgG receptor on the surface. The total thickness measured for these two cases were 45 ± 26 and 46 ± 23 Å, respectively.

The coating and exposure procedures were subsequently performed on live sensors using biotinylated anti-dog IgG as the receptor coating, and dog-IgG as the analyte. As shown in Fig. 5, the resonant frequency of the miniature resonator is decreased by the subsequent additions of the avidin, biotinylated receptor protein, and the IgG analyte. This initial result indicates that a similar receptor application approach and exposure protocol will yield similar results for the detection of other proteins.

4.2. Detection of interleukins

Cytokine detection in patients has attracted the attention of intensive care surgeons. A trauma team at the Brigham and Women's Hospital (BWH) identified several interleukins (IL-1, -2, -4, -6, -8, -10) whose elevated concentrations in blood or urine have been found to correlate positively with multiple organ failure (MOF) rates in trauma patients. [18] Real-time determination of the concentrations of amino acids, antioxidants, enzymes, and metabolites were also considered relevant to trauma diagnosis. We chose to first focus on interleukin detection because protein receptor molecules were readily available, and it was determined that larger molecules would be easier to detect with the sensor. For proof-of-concept demonstration of the cytokine receptor immobilization chemistry, PBS mixed with calibrated concentrations of target cytokine, recombinant human IL-6, was used to test the sensor rather than blood serum.

The result of our first experiment for the detection of IL-6 in buffer solution using a μCANARY sensor activated with anti-IL-6 receptor is shown in Fig. 6. For this work, we used the same "generic" protein attachment method through an avidin anchor molecule shown to work with dog-IgG detection. The sensor frequency shift response is shown for two IL-6 exposure concentrations, 8 and 160 ng/ml, in which the latter induced a much greater frequency shift. This result shows that the sensor produces measurable response to IL's with sensitivity on the order of ng/ml (∼50 pM) or better, and is the beginning of building a dose–response curve for a quantitative assay.

Through our discussions with the BWH team, it became evident that the ability to simultaneously monitor a host of cytokines would have tremendously higher predictive value

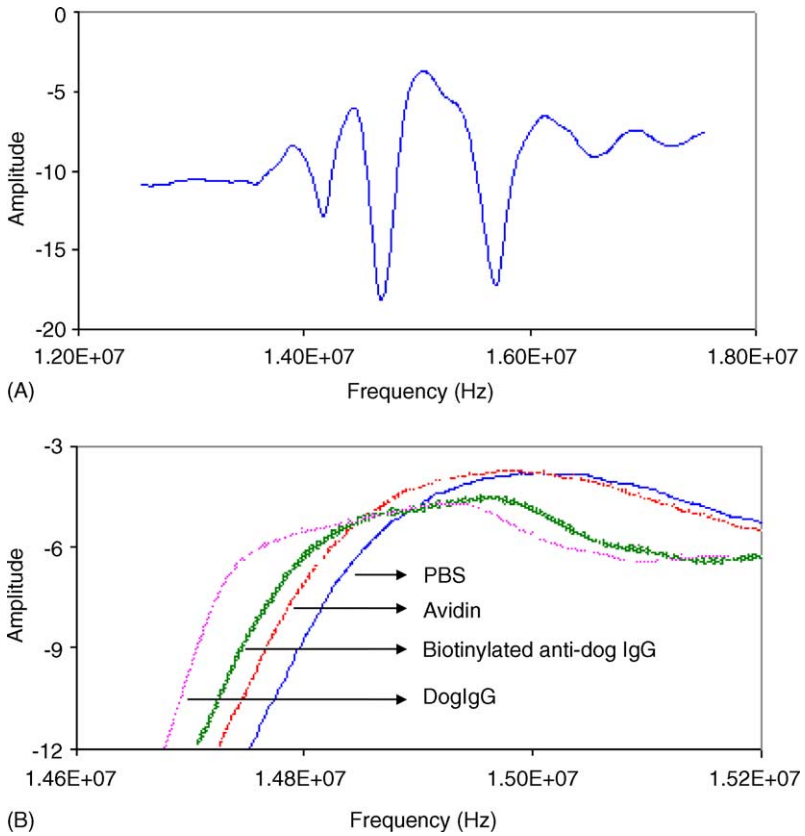


Fig. 5. (A) μCANARY resonant frequency response when exposed to PBS. The peak at ~15 MHZ is the major resonance mode that is used to monitor the biointeractions on the sensor surface, and (B) μCANARY frequency readings when sequentially exposed to PBS, avidin, anti-dog IgG biotin conjugate, and dog-IgG. To increase resolution, the sensor was monitored within a 500 kHz range at the major resonance peak. Lower resonant frequency indicates attachment of molecules to the sensor surface.

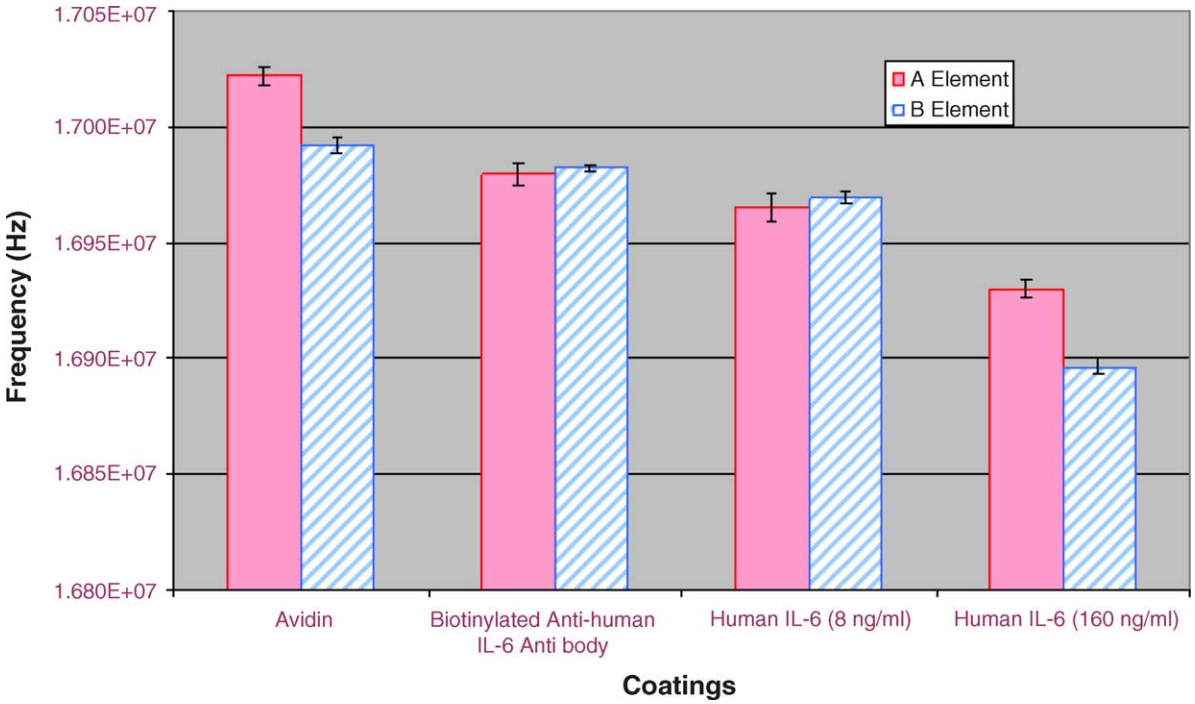


Fig. 6. Frequency response of two sensing elements (A and B) within a 2-element μCANARY to recombinant human interleukin-6. The frequencies shown are the average value of 10 readings. In the experiment, conditions were not optimized to determine the sensitivity of the sensor response. Estimate of the sensitivity was made based on the relative signal level above experimental noise.

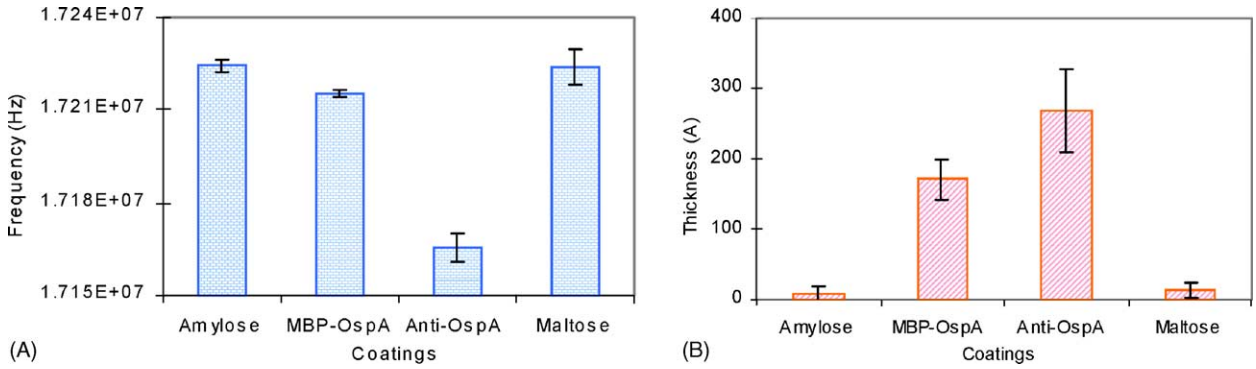


Fig. 7. μCANARY resonant frequency response (A) and ellipsometry measurements (B) for detection of anti-OspA antibody. With the immobilization of each coating, more mass was added to the sensor, which induced the decrease in frequency signal and the increase in coating thickness on the sensor surface. When maltose was added to the sensor after detection, the sensor surface was re-generated. Error bars represent the standard deviation of five measurements.

than measurement of a single protein. As a result, a sensor array would provide the greatest benefit and it became necessary for us to consider miniaturization of the sensor to enable integration of several assays into the same silicon chip. We then pursued the development of a miniature sensor that could be configured into an array of eight elements.

4.3. Detection of anti-OspA and disease susceptibility testing

To explore the usefulness of μCANARY sensors for detection and quantification of antibody in clinical samples, and for use as a vaccine development tool, Draper and NEMC collaborated in a project that involved the testing of a vaccine for Lyme disease.[1] The protein analyte for this work was antibody to an outer surface protein (OspA) of the spirochetal bacteria, *B. burgdorferi*, that cause Lyme disease. Because antibodies to intact OspA and to a particular OspA epitope are protective in animals and humans, OspA is the currently available vaccine for Lyme disease protection. NEMC has serum samples obtained from volunteers who either received the active vaccine or a placebo. We plan to use the μCANARY sensors for detection and quantification of antibody in these clinical samples to determine the feasibility of using this format for predicting which individuals are effectively protected and which individuals are susceptible to Lyme disease.

As proof of concept demonstration, we started with the detection of laboratory-prepared anti-OspA sample. For this work, it was our goal to apply a monolayer of OspA to a μCANARY sensor, so that it would detect anti-OspA in a serum test solution. A series of steps were required in order to properly attach the receptor protein to the sensor. A sugar, amylose, was used to bind a maltose binding protein-complexed OspA to the surface, which utilized the binding between amylose and MBP. Once the MBP–OspA complex was immobilized on the μCANARY, it was ready to detect anti-OspA. Fig. 7A shows the aqueous resonant frequency of a μCANARY after the MBP–OspA receptor coating was applied, and after the sensor was exposed to a 200 ng/ml anti-OspA (affinity purified at NEMC) in saline solution. For this first experiment, a ~50,000 Hz frequency shift was measured when it captured and detected anti-OspA, indicating that the sensor should be capable of measuring much lower concentrations. Ellipsometry measurements were also performed to confirm the sequential binding of each coating layer to the sensor (Fig. 7B). Since it was difficult to set up the wired sensor chip for both frequency and ellipsometry measurements at every step, a different chip with the same sensor structure but without electronics was made as a simulated chip for the ellipsometry measurements.

Since the μCANARY sensors eventually can be made very inexpensively using similar but much simpler silicon fabrication methods as those used in today's computer industry, we anticipate that disposable μCANARY chips will be affordable. However, in some situations where large numbers of chips are not available or when sensors are used in vivo, regenerable sensors might become desirable. To this end, experiments were performed to demonstrate the ability to regenerate a chip after a positive exposure. By washing the chip that had been exposed to anti-OspA (Fig. 7) with a maltose-rich solution, the maltose binding protein in the MBP–OspA molecule detaches from the amylose on the chip's surface, and binds with the maltose in solution since MBP binds to maltose more strongly than it does to amylose. By rinsing the chip in this manner, we have shown that the device can be returned to an amylose-coated condition repeatedly, and that the chip can therefore be re-used. Fig. 7 shows that the removal of MBP–OspA by this method can

[1] Lyme disease is a tick-born bacterial infection. The bacterium, *B. burgdorferi*, lives in the gut of certain tick species, and is transferred to humans through tick bites. *B. burgdorferi* expresses proteins on its outer surface which can be recognized by the immune system to elicit an immune response. One of the most plentiful of these proteins is OspA. The vaccine for Lyme disease is recombinant OspA so that the immune system will produce the antibody to OspA (i.e. anti-OspA). The effectiveness of the vaccination can be determined by measuring the concentration of anti-OspA in a blood sample.

be measured with the μCANARY. The regeneration method can be extended to other assays, in which a relatively weaker binder is used as a capture probe and a stronger binder is used as a regeneration agent.

For many chemical and biological sensors, detection of analyte of interest in a laboratory-prepared, relatively "clean" sample (i.e. buffer solution spiked with analyte) is not very challenging. However, when the sample matrix is as complex as blood, where the target protein is overwhelmed by the presence of many other proteins, sensitivity, and selectivity become a great concern. To test the ability of the μCANARY sensor to capture the target protein in the presence of others, experiments have been planned to challenge the sensor with patient's serum samples. The coating procedure developed above for the laboratory-prepared samples could be used.

4.4. Detection of intact bacteria

After the successful detection of outer surface protein A of the Lyme disease bacteria, *B. burgdorferi*, more tests followed to further demonstrate the ability of the μCANARY sensor to detect whole bacteria and to measure sensor selectivity through exposure to *B. burgdorferi* mutants engineered not to express OspA. The in vitro cultivated strains *Borrelia* N40, in which OspA was abundantly expressed on the surface, and *Borrelia* 7 × 297, which did not express OspA, were chosen for the proof-of-principle experiment. Results of the experiment are shown in Fig. 8.

As shown in Fig. 8, the receptor ligand monolayer was attached to the sensor surface through sequential exposure to solutions containing avidin, biotinylated human IgG, and human anti-OspA. A resonance frequency decrease was observed for each exposure. After immobilization of anti-OspA, the sensor was ready for *Borrelia* detection by binding to OspA on the bacteria. When the sensor was challenged by the presence of 10^6 *Borrelia* 7 × 297 (a high concentration of bacteria sample was used to ensure binding occurred), no significant signal (4000 Hz, within the experimental error of 5000 Hz) was observed since the intended recognition protein, OspA, was absent from the bacteria. Meanwhile, when the sensor was exposed to the same amount of N40 bacteria, a significant frequency shift (27,000 Hz) was recorded, which illustrated that the sensor specifically detected the presence of N40.

The sensor was then regenerated by extreme pH shift (pH 2 then pH 12, repeat three times) to remove anti-OspA before again being exposed to a neutral environment (pH 7.4 in PBS). Now with the presence of either 7 × 297 or N40 bacteria, no detection on μCANARY was observed. When anti-OspA was subsequently immobilized on the sensor, N40 was again detected by μCANARY (Fig. 8). To confirm that the sensor indeed detected the wild-type *borrelia* N40 and not *Borrelia* 7 × 297, confocal microscopy photos were taken of sensors that had been exposed to the two different strains of the bacteria (Fig. 9). The results indicated positively that the μCANARY sensor could be used to detect intact microorganisms, and with the appropriate receptors available it is also possible to identify different strains of the same bacteria using a sensor array. Using the same principle, we have also detected *B. subtilus* spores [19] using a μCANARY sensor.

The currently available methods for the detection of bacteria in environmental and biological specimens involve the traditional methods of direct staining for visualization of bacteria, serologic techniques that detect antigens from the bacteria, or evidence of prior infection by an antibody response to bacterial antigens. There are also a wide array of

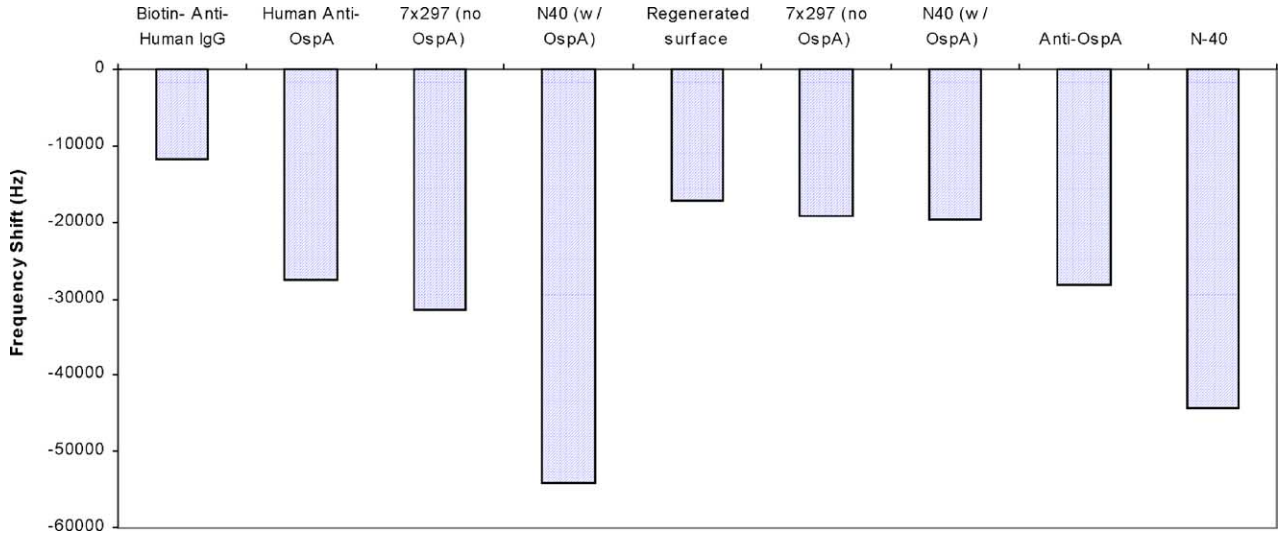


Fig. 8. Preparation of μCANARY sensor with affinity ligand protein anti-OspA for detection of wild-type *B. burgdorferi* (strain N40) expressing OspA on its outer surface. Mutant strain 7 × 297, which does not express OspA, is not detected. The sensor surface is subsequently stripped of anti-OspA using pH shock. The sensor without anti-OspA detects neither N40 nor 7 × 297. When anti-OspA is immobilized on the sensor, it again detects the N40 strain.

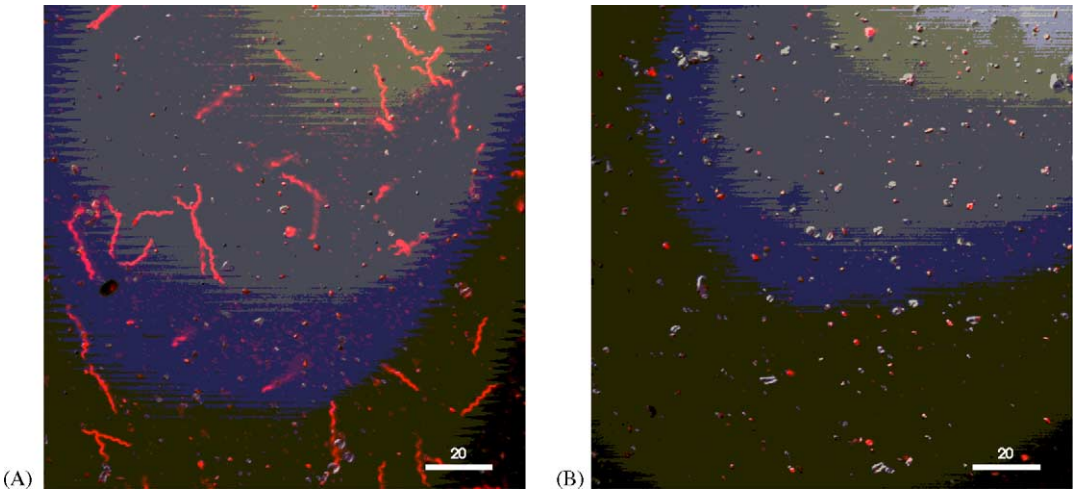


Fig. 9. Confocal microscopy pictures for μCANARY sensors that have anti-OspA coated on the surface and have been exposed to *Borrelia* N40 (A, Nile-red labeled spirochet, with OspA on outer coat) and *Borrelia* 7 × 297 (B, without OspA on outer coat), respectively. The scale shown in the pictures is 20 μm. The frequency shifts observed for A and B are ∼27 and ∼4 kHz, respectively.

culture techniques, and the most recent additions are nucleic acid based molecular techniques (e.g. polymerase chain reaction based amplification methods). Our new bacterial detection and identification system could prove to overcome the limitations of these detection methods. Further investigation is warranted to devise an array-sensing scheme where different receptor coatings can be applied to identify different bacteria or different strains of the same bacteria.

5. Conclusions

In conclusion, a miniature FPW sensor and sensor array have been used to perform several different types of biological assays. An electronic addressing, excitation, and detection system has been built to enable high sensitivity monitoring of resonant frequency shifts, and to subtract common-mode effects. A receptor coating protocol has also been developed for the covalent attachment of antigens and/or antibody molecules to the sensor, and preliminary results have been obtained demonstrating the ability of the sensor to detect antibodies or specific biological analytes in aqueous solution. In addition, affinity ligand receptors which target proteins on the outer surface coat of intact microorganisms have been immobilized onto the sensor membrane, which enable the sensor to recognize *B. burgdorferri* bacteria. Detection selectivity is demonstrated through the recognition of wild-type *B. burgdorferri* that express a particular outer surface protein, while mutated organisms with the surface protein deleted are not detected. Means have also been developed for chemically detaching detected microorganisms from the chip, and subsequently regenerating the sensor surface. The experiments described in this report are a prelude to the demonstration of larger biosensor arrays that will be capable of measuring the interaction between many ALRs with a test sample for applications that include proteomics, diagnostics, and high throughput screening.

Acknowledgements

This project was jointly sponsored by the Department of the Army, Cooperative Agreement DAMD-99-2-9001 and Draper Laboratory. The content of this paper does not necessarily reflect the position or the policy of the government, and no official endorsement should be inferred. JWP and BTC gratefully thank Dr. Juan Carlos Puyana and his team for helpful discussion about detecting proteins critical to ICU patients using the μCANARY technology. The authors wish to thank Rick Rogers at Harvard Medical School for providing the confocal microscopy images. Connie Cardoso and the staff within the Draper MEMS fabrication facility are acknowledged for their valuable assistance in device fabrication. AlN piezoelectric thin films were provided through MIT by Prof. Rafael Reif and Draper Fellow Wendy Mao. JWP and BTC acknowledge the assistance provided by Jeff Pierce, Chris Mooney, and Jorge Robellar for writing the data acquisition and analysis software, Jeff also for taking some sensor test data, Jay Duffner and Joshua Jondro for writing the BioDot data acquisition program for the 8-element μCANARY sensor, and Chris Dube for his efforts on installing the BioDot system.

References

[1] (a) International Human Genome Sequencing Consortium, Nature 409 (2001) 860–921;
(b) J.C. Venter, et al., Science 291 (2001) 1304–1351.
[2] K.L. Michael, L.C. Taylor, S.L. Schultz, D.R. Walt, Anal. Chem. 70 (7) 1242–1248.

[3] R.L. Edelstein, C.R. Tamanaha, P.E. Sheehan, M.M. Miller, D.R. Baselt, L.J. Whitman, R.J. Colton, Biosens. Bioelectron. 14 (10–11) 805–813.
[4] T.A. Taton, C.A. Mirkin, R.L. Letsinger, Science 289 (5485) 1757–1760.
[5] R.L. Rich, D.G. Myszka, Curr. Opin. Biotech. 11 (1) 54–61.
[6] H. Arwin, Thin Solid Films 313 (1998) 764–774.
[7] P.J. Skrdla, S.S. Saavedra, N.R. Armstrong, S.B. Mendes, N. Peyghambarian, Anal. Chem. 71 (7) (1999) 1332–1337.
[8] B. Cunningham, M. Weinberg, J. Pepper, C. Clapp, R. Bousquet, B. Hugh, R. Kant, C. Dely, E. Hauser, Sens. Actuat. B 73 (2001) 112–123.
[9] S.W. Wenzel, R.M. White, Sens. Actuat. A A21–A23 (1990) 700–703.
[10] J.W. Grate, S.W. Wenzel, R.M. White, Anal. Chem 63 (1991) 1552–1561.
[11] R.M. White, S.W. Wenzel, Plate-Mode Ultrasonic Sensor, US Patent 5,129,262 (July 1992).
[12] Q.Y. Cai, J. Park, D. Heldsinger, M.D. Hsieh, E.T. Zellers, Sens. Actuat. B 62 (2000) 121–130.
[13] M.S. Weinberg, B.T. Cunningham, C.W. Clapp, J. MEMS 9 (3) 370–379.
[14] (a) G.M. Whitesides, P.E. Laibinis, Langmuir 6 (1990) 87;
(b) L. Yan, W. Huck, X.M. Zhao, G.M. Whitesides, Langmuir 15 (1999) 1208–1214;
(c) R.C. Horton Jr., T.M. Herne, D.C. Myles, J. Am. Chem. Soc. 119 (1997) 12980–12981;
(d) M.E. Meyerhoff, C. Duan Mikrochim, Acta 117 (1995) 195–206.
[15] P. Hsieh, R. Reif, B. Cunningham, DC magnetron reactive sputtering of low stress aluminum nitride piezoelectric thin films for MEMS applications, in: Proceedings of the 1998 Material Research Society Fall Meeting, Boston, MA.
[16] R.P. O'Toole, S.G. Burns, G.J. Bastiaans, Thin aluminum nitride film resonators: miniaturized high sensitivity mass sensors, Anal. Chem. 64 (1992) 1289–1294.
[17] F. Laermer, A. Schilp, Method of Anisotropically Etching Silicon, US Patent #5,501,893.
[18] Private Communication with Dr. J.C. Puyana and the Trauma Team, Brigham and Women's Hospital, Boston, 1999.
[19] J. Pepper, R. Noring, A. Petrovich, R. Bousquet, J. Brady, B. Hugh, M. Klempner, B. Cunningham, *B. subtilus* Strain PY79 was generously provided by Dr. Adam Dricks at Loyola University, Unpublished results.