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The George W. Woodruff School of Mechanical Engineering
Atlanta, GA 30332-0405 USA

November 22, 2005

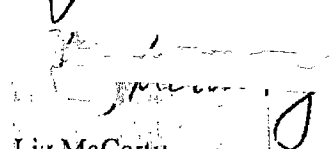
National Institutes of Health (NIH)
9000 Rockville Pike
Bethesda, Maryland 20892

Dear Sir/Madam:

Enclosed please find the final technical report for NIH grant 5R21EB000767-02:
IMMUNOASSAYS ON PLASTIC CANTILEVERS.

Thank you,


Jonathan W. S. Colton
Professor of Mechanical Engineering


Liz McCarty
Program Officer, Office of Sponsored Programs.

Final Report

NIH GRANT 5R21EB000767-02

IMMUNOASSAYS ON PLASTIC CANTILEVERS

9/30/2002 – 8/31/2005

**Georgia Institute of Technology
Office of Sponsored Programs
505 Tenth Street
Atlanta, GA 30332-0420**

November 22, 2005

PIs: Prof. Jonathan Colton, Prof. Larry Bottomley

IMMUNOASSAYS ON PLASTIC CANTILEVERS

Final Report to NIH, Grant 5R21EB000767-02

A summary of the proposal is as follows:

Summary: Biosensor devices based on the nanomechanical motion of microcantilevers are an emerging sensor platform. Molecular adsorption on a resonating microcantilever shifts its resonance frequency; resonance shifts are correlated with changes in cantilever mass. Interaction of analytes with molecules tethered to one surface of the cantilevers induces differential surface stress that causes cantilever bending. High selectivity in response is achievable through incorporation of biomolecular recognition elements as thin film coatings on the cantilever. The long-range goal of this research is to develop a commercial, revolutionary microcantilever platform technology for sensing applications requiring sensitive, specific, quantitative and multiplexed detection of fluid-borne analytes. The immediate goal is to develop a plastic microcantilever platform technology for sensitive, specific, quantitative, multiplexed, and label-free detection of proteins present in complex mixtures. These will be fabricated using low cost, high-yield, mass-production polymer processing techniques, such as micro-injection molding. The inherent advantages of this method include the ease with which the properties of the cantilever can be tuned to meet its intended application and significantly reduced fabrication costs (materials, tooling and labor). Our specific aims are focused on optimizing production methods for fabricating plastic microcantilevers, optimizing the cantilever design, and developing plastic microcantilever-based enzymatic assays. These three aims have been met to various degrees.

The specific aims from the proposal are as follows:

<i>Specific Aim 1: Explore methods of fabricating plastic microcantilevers</i>
Design of Prototype Thermoplastic Cantilever Molds
Process variable optimization
Microcantilever Characterization
<i>Specific Aim 2: Optimize cantilever design</i>
Cantilever Design and Fabrication Modifications
Modeling and Simulation of Fabrication and Use
<i>Specific Aim 3: Develop plastic microcantilever-based immunoassays</i>

The work accomplished to meet these aims described below.

Specific Aim 1: Explore methods of fabricating plastic microcantilevers

Specific Aim 2: Optimize cantilever design

Plastic cantilevers have been fabricated from polystyrene (a well characterized polymer, heavily used in bioassays) via injection molding with our micro-injection molding machine (see figure 1 - the figure on the left shows four cantilevers; the figure on the right is an enlargement

of one end of one cantilever). Figure 2 shows a side view of the cantilevers. The base part is used to hold the cantilever in the AFM, which serves as the detection system. The molds were machined using a purpose-built micro-milling machine (see figure 3) into steel gage blocks. The milling machine is approximately 250 mm on a side. The solid carbide milling tools used were 75 microns in diameter and rotated at 50,000 revolutions per minute. Steel gage blocks were used to assure the very flat surfaces (smoothness on the order of 10s of nanometers) needed so the mold cavities would not leak. Steel molds assured a robust manufacturing process, as opposed to aluminum or silicon, which are prone to wear and damage. We used an innovative mold design which included springs so the molds had rotational degrees of freedom so that their surfaces would meet flush. Optimal processing conditions were determined by experimentation (references 1, 2, 3, 10, 12, and 13 discuss the details of the injection molding process, including the design and production of the molds and the equipment used). While we cannot honestly say the process is flawless, we are able to make hundreds of parts reproducibly and with minimal differences in their dimensions and mechanical behavior. For example, the standard deviations of the natural frequencies of the microcantilevers were less than 3%. The parts have dimensions 2- 45 microns thick, 77-168 microns wide, and 374 - 755 microns long and tolerances much less than 1 micron. The mechanical behavior of the parts was characterized, including the Q factor and the spring constants. These beams have stiffnesses between 0.01 and 10 N/m and Q factors between 10 and 70. These results show that we can reproducibly make cantilevers with the correct mechanical properties and tolerances needed for biochemical sensing. References 1, 2, 3, 4, 6, 8, 9, 10, 12 and 13 discuss the characterization and mechanical properties of the beams.

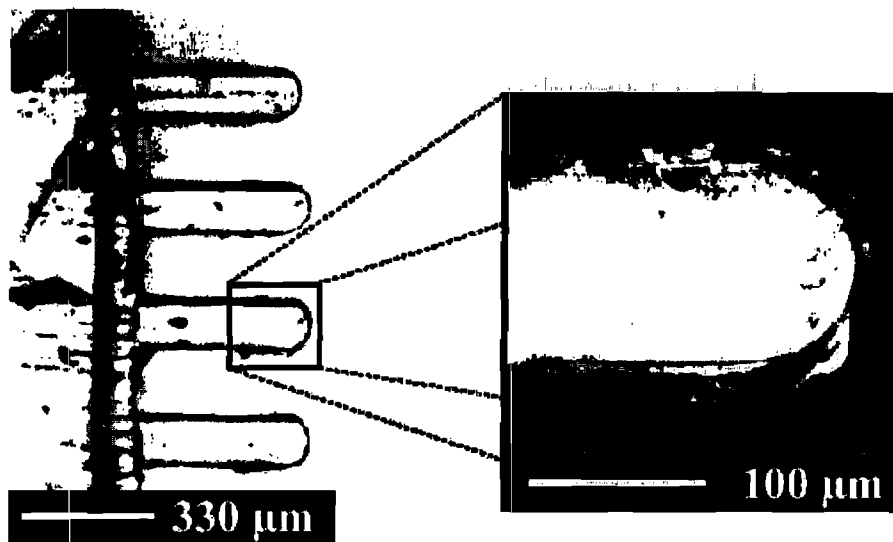


Figure 1. Optical micrograph of polystyrene cantilevers.

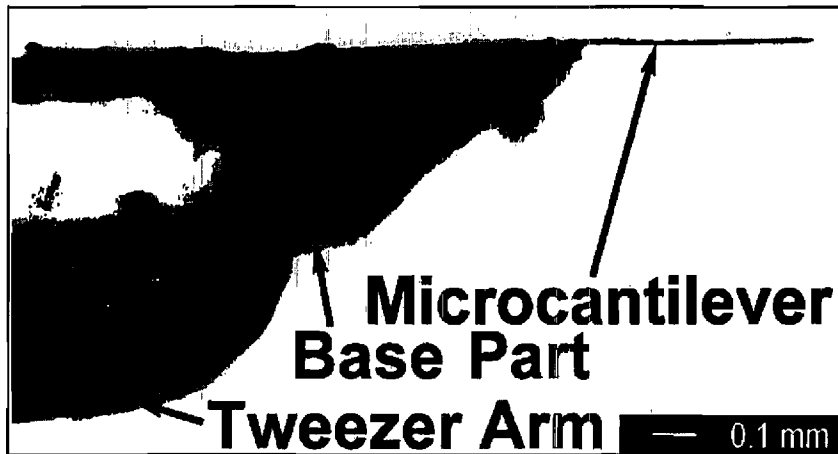


Figure 2. Side view of cantilevers.

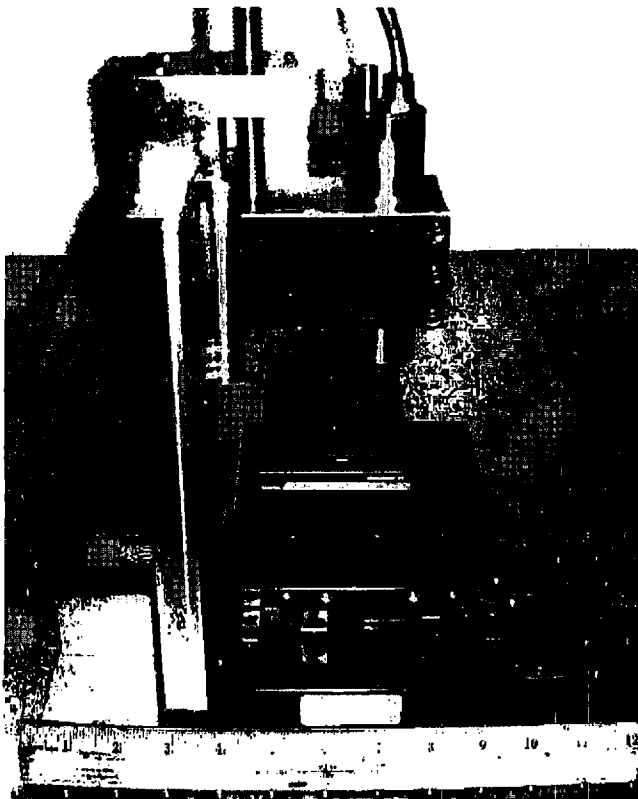


Figure 3. Micromilling machine

In addition, we produced plastic scanning probe microscope probes. These are plastic cantilevers with small tips on the ends (see figure 4). They can be used for interrogating surfaces, as conventional SPM probes, but have the advantages associated with polymers, as discussed in this report. These probes have been shown to image micron high steps with similar accuracy as commercial silicon nitride tips (see figure 5). This opens a whole are of SPM

microscopy to explore. Georgia Tech has applied for a patent on this technology. Reference 7 provides details of the manufacture and testing of these probes.

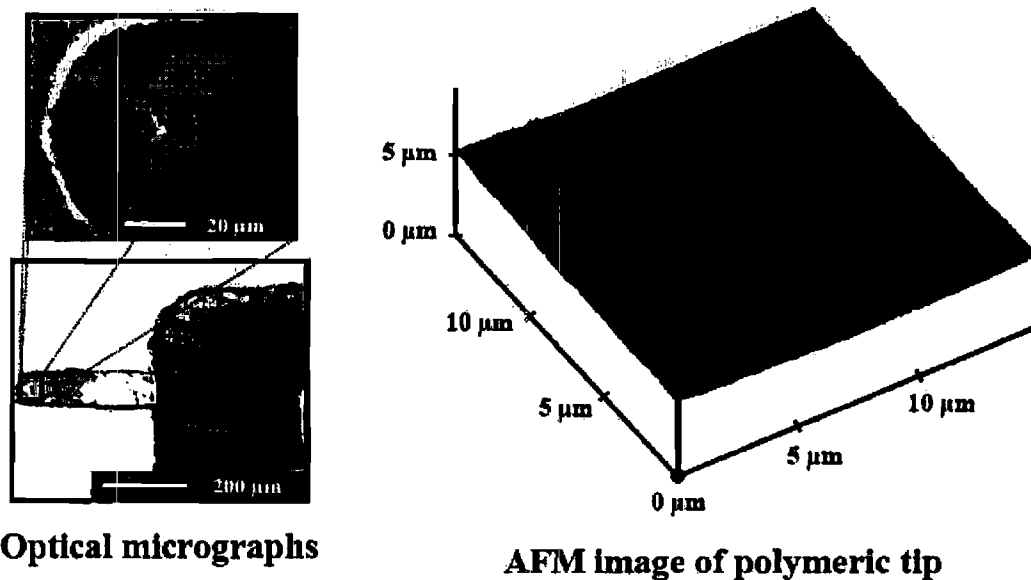


Figure 4. Plastic scanning probe microscopy probes. AFM image of tip on right.

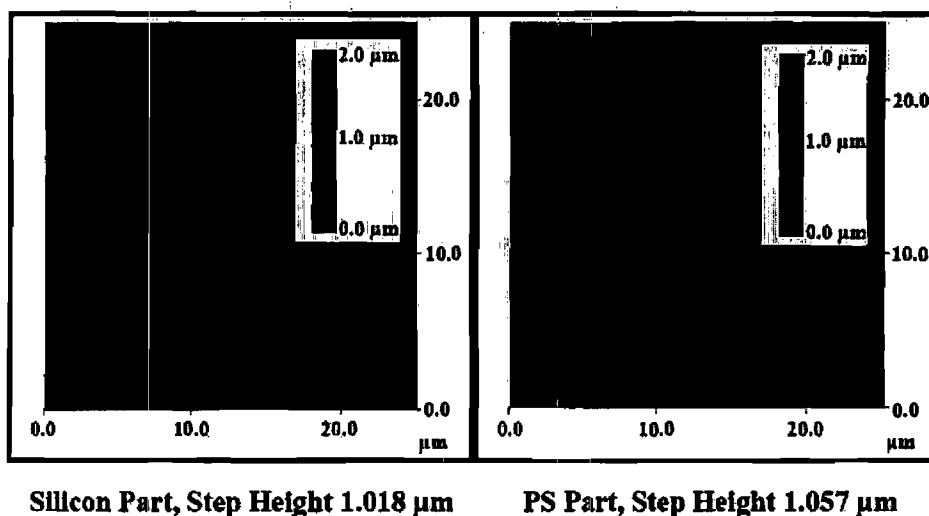


Figure 5. Imaging of a 1 micron high grating step (left - silicon nitride tip, right – plastic tip)

Specific Aim 3: Develop plastic microcantilever-based immunoassays

At the time of proposal submission, we anticipated that the plastic microcantilever arrays would be suitable for detection of antigen-antibody binding. During the development phase

(design and fabrication of plastic cantilevers) we identified a more promising application of plastic microcantilever arrays for biodetection – enzymatic assays. We have successfully obtained results that provide proof of concept that microcantilevers can be used to measure enzymatic action, at least for enzymes that by their action on the substrate cause a change in the surface stress. In this case, however, the deflection may be upward or downward, depending upon whether the surface stress is increased or decreased. The intrinsic advantage of microcantilever-based enzyme assays is best illustrated in Figure 6. When the cantilever is coated with a non-substrate for the enzyme in question, no deflection is anticipated. If non-specific adsorption of the enzyme occurs, then downward deflection will be observed. Thus, by simple variation of the substrates attached to various cantilevers in the array, high fidelity detection of the presence of a particular enzyme(s) is possible, especially when the array is subjected to a complex mixture (e.g., cell lysate). A second intrinsic advantage is that this protocol can also be used to detect enzyme inhibition as well as enzyme activity. Figure 6 depicts the expected deflection when the cantilever is coated with the appropriate enzyme substrate but an inhibitor is present in solution. This particular application of microcantilever array sensors has broad implication in drug discovery and high throughput screening applications. The ability to monitor enzyme function and inhibition in complex mixtures will be a “killer application” of this technology.

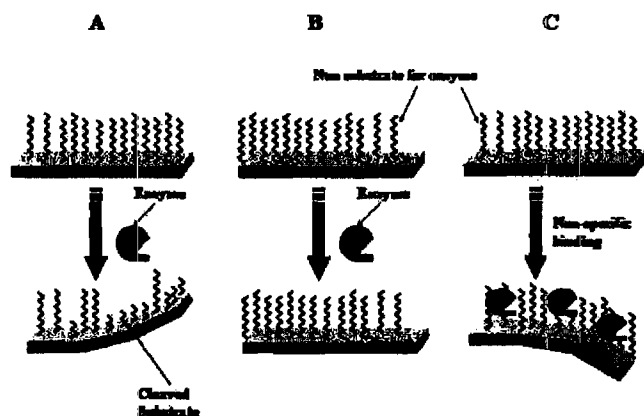


Figure 6: Schematic of Microcantilever-based enzyme assay

To measure the deflection of multiple cantilevers (each of which could be coated with a different substrate), we use a Veeco Instruments Metrology Division Scentris system. This instrument enables simultaneous measurement of the deflection of up to eight parallel cantilever beams in air or in fluid. We use a Packard biofluidics dispenser system to coat each cantilever in the array with a different enzymatic substrate using standard polypeptide attachment chemistries. Thus, we are able to carry out the development of a novel protease sensor.

We will consider the goals of this third aim met if we accomplish the high fidelity detection of enzymatic action and its inhibition in complex fluid mixtures. To achieve high fidelity detection of protease action, the rate of cantilever deflection due to mass loss must be proportional to enzyme (and substrate) concentration and be commensurate with the rates observed by traditional bioanalytical methods for surface-confined substrates. In addition, the

rate of cantilever deflection must be impeded in proportion to the concentration of enzyme inhibitor present in solution. Also, detection of enzyme action and inhibition must be established in mixtures containing other enzymes that do not act on the substrate.

Initial studies were devoted to comparing the performance of plastic microcantilever sensors to their silicon counterparts for both air-borne and fluid-borne analytes. Sensing studies were performed monitoring both cantilever deflection and frequency shift detection modes. Pertinent results are summarized below and in references 4, 10, 11, 12, and 13.

Detection of Humidity. Increases in mass should result in a decrease in cantilever resonance frequency. To compare the sensitivities to mass changes for polystyrene cantilevers with silicon cantilevers, the frequency of the two cantilever types was correlated with increases in the ambient humidity. Increases in cantilever mass results from adsorption of water onto the surface of the cantilever.

Laboratory air was pumped into the Scentris system fluid flow cell containing either a gold coated, four-beam, polymeric microcantilever chip or a gold coated eight-beam silicon cantilever chip. The relative humidity of the air stream was controlled by mixing laboratory air with water-saturated air prior to delivery into the cell. The humidity level was measured with a hygrometer placed at the exit of the cell. Microcantilever resonance was measured as a function of time and was continuously recorded while the air diffused into the flow cell. Figure 7 depicts the response of the cantilevers to increasing humidity and proves that the response of polystyrene cantilevers parallels that of their silicon counterparts.

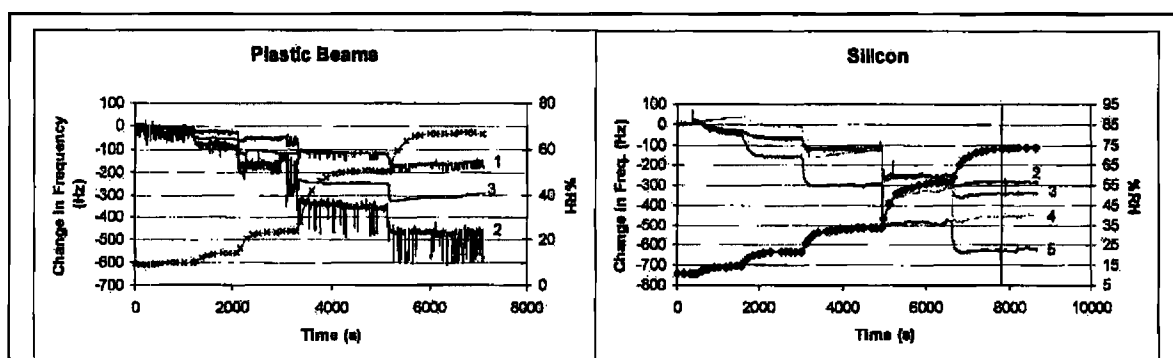
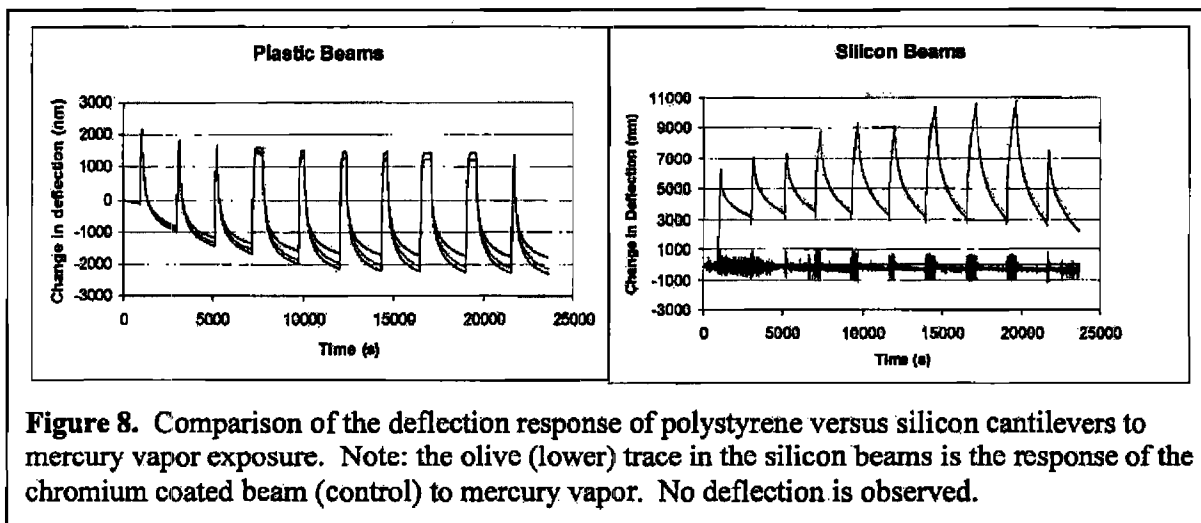


Figure 7. Comparison of the frequency shift response of polystyrene and silicon microcantilevers as the relative humidity in the flow cell increased with time.

Detection of Mercury Vapor. Similarly, the performance of polystyrene cantilevers for detection of mercury vapor in air streams was compared to that of silicon cantilevers. Detection of airborne mercury is one of the benchmarks for commercial cantilever detection devices currently on the market. Mercury vapor was introduced to the flow cell in a manner parallel to that used for detection of changes in ambient humidity. Cantilevers with thin gold coatings on one side were used as active sensors; cantilevers with thin coatings of chromium on one side were used as controls. Mercury forms an amalgam with gold. Thus, when these cantilevers are exposed to mercury vapor, chemisorption into the gold film results in an increase in the volume

(and mass) of the gold film producing a stress on the beam that causes it to deflect. Figure 8 depicts the change in deflection of the cantilevers to repetitive injections of mercury vapor into the cell. Note that the formation of the gold-mercury amalgam is reversible. When mercury vapor is present in the cell, the deflection increases; when mercury vapor is swept from the cell, the deflection returns to the baseline. The data presented in Figure 8 prove that the response of polystyrene cantilevers for detection of mercury parallels that of their silicon counterparts.



Ethanethiol Vapor Detection. A vapor-borne ethanethiol was diffused into the Scentris system fluid flow cell (laboratory air-filled) where a gold coated, four-beam, polymeric microcantilever part was located. The microcantilever deflection as a function of time was recorded while the thiol diffused into the flow cell. At steady-state (see Figure 9), the four different microcantilevers showed surface stress values of roughly 75 mN/m, 75 mN/m, 83 mN/m, and 77 mN/m. These values are in good agreement with each other and with previously published reports of ~82 mN/m obtained using the same experimental methods. To gauge accuracy and repeatability of the monolayer formation-induced surface stress experiments, the thiols were flowed over ten cantilever parts (each with four microcantilevers) made from PS, PP, and NN6 (a total of 30 parts with a total of 120 cantilevers). Steady-state surface stress values were obtained for each of the parts of 74 mN/m, 83 mN/m, and 64 mN/m for the PS, PP, and NN6 parts, respectively) show that the steady-state surface stress is in reasonable agreement (for the same thiol at similar concentration) with the literature values (82 mN/m). These results demonstrate that injection-molded polymeric microcantilevers are feasible deflection-based, vapor-phase, gold-thiol bonding sensors which produce experimental results in agreement with literature values obtained with the same experimental methods. This is the first step to proving that plastic cantilevers function as sensors.

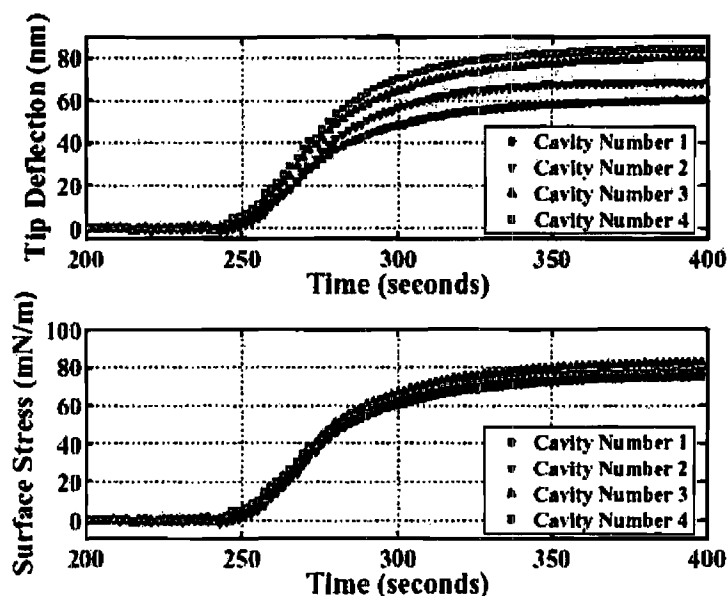


Figure 9. Surface Stress Response

Detection of Ethanol in Flowing Streams. To assess the response of polystyrene cantilevers to fluid-borne analytes, the cantilever chip was mounted in the fluid cell. The deflection of each beam was monitored as a function of time as the composition of the fluid flowing through the cell was systematically changed using flow injection techniques. Figure 10 depicts the cantilever during repeated injections of solutions containing 10, 30, and 50% EtOH, respectively into the fluid stream. Note: the direction of cantilever deflection is opposite of that observed in mercury vapor studies described above. This observation indicates that ethanol is partitioning into the plastic, increasing its volume and producing an upward deflection of the cantilever. No observable deflection was observed for silicon cantilevers. This finding also suggests a new paradigm for microcantilever detection – the chemically sensitive layer may be the cantilever itself!

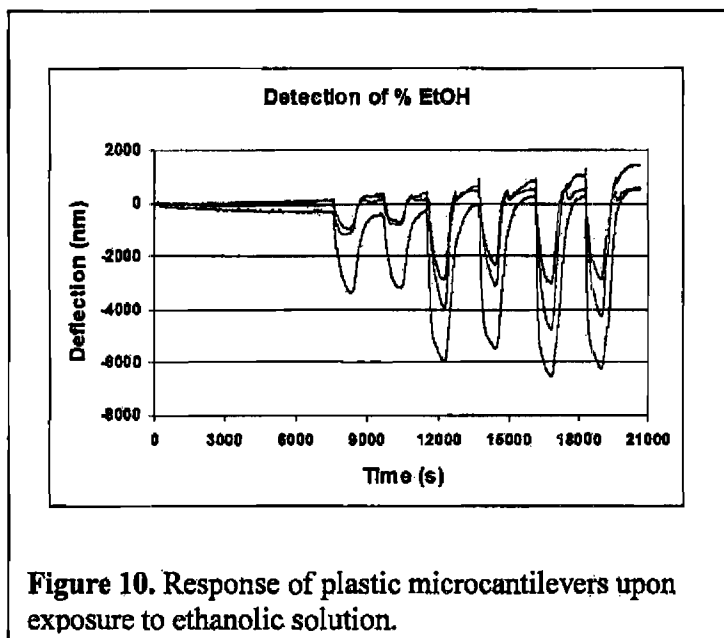


Figure 10. Response of plastic microcantilevers upon exposure to ethanolic solution.

Within the timeframe of this research grant, we were not able to accomplish a successful enzymatic assay on plastic microcantilevers due to the length of time required to perform the necessary preliminary steps. We have laid the ground work for enzymatic assays on plastic cantilevers. In our future work, we will focus on providing proof of concept for microcantilever-based enzymatic assays. A schematic of our design concept is provided in Figure 6 for a protease. Enzymes from this class cleave a substrate at a specific location when the substrate possesses the correct structural or molecular recognition element. Panel A depicts the expected response of the cantilever following immobilization of a protease substrate onto one side of the cantilever and incubation with the protease. Upward deflection of the cantilever results from a decrease in mass, a decrease in the surface stress imposed by the substrate layer, and an increase in solvation. To achieve high fidelity detection of specific enzyme action, simultaneously run control experiments are required. Panel B depicts the expected deflection when a cantilever coated with a non-substrate is exposed to the protease. Panel C depicts the expected deflection when the protease (or other biomolecule in the test solution) non-specifically adsorbs onto the cantilever. Downward deflection is expected in proportion to the mass increase of the cantilever resulting from the adsorption of biomolecules. Thus, high fidelity detection of the specific protease is achievable in the absence of non-specific binding by comparative measurement of the deflection in panels A and B. Similarly, in the presence of specific binding, high fidelity detection of the specific protease is achievable in the absence of non-specific binding by comparative measurement of the deflection in panels A and C. Experiments currently underway utilize double-stranded DNA oligomers as the substrate/non-substrate (depending upon sequence) and three different endonucleases as the test enzyme. The extent of non-specific binding is controlled by the concentration of magnesium ion, an important co-factor in the cutting action of the endonuclease.

Summary

We have successfully designed and fabricated plastic microcantilevers for BioMEMS sensing using economical, mass production techniques. Characterization of these microcantilevers shows them to have the proper mechanical and chemical properties needed for use as BioMEMS sensors. They have been shown to work as sensors for water vapor, mercury vapor, ethanethiols, and ethanolic solutions. This work has set the foundation for the use of plastic microcantilevers as sensors for enzymatic assays.

In addition, we have developed, fabricated and tested plastic SPM probes, which opens a new area of SPM activity, as their properties can be easily tailored by changing their material, without changing their geometries.

Publications

The following 13 papers and presentations have resulted, at least in part, from this project.

1. McFarland, A.W., Poggi, M.A., Bottomley, L.A., and Colton, J.S. "Production and Characterization of Polymer Microcantilevers," Review of Scientific Instruments, No. 75, Vol. 8, 2756-2758 (2004)
2. McFarland, A.W., Poggi, M.A., Bottomley, L.A. and Colton, J.S., "Injection Moulding of High Aspect Ratio Micron-Scale Thickness Polymer Microcantilevers," Nanotechnology, Vol. 15, No. 11, 1628-1632 (2004)
3. Bottomley, L.A., McFarland, A.W., Poggi, M.A., and Colton, J.S., "Polymeric Microcantilevers for Sensing Applications," 227th American Chemical Society National Meeting, Anaheim, March 28-April 1, 2004
4. McFarland, A., Colton, J.S., Poggi, M., and Bottomley, L.A., "Plastic BioMEMS Sensors," Proceedings of ANTEC '04, 3717-3721, Society of Plastics Engineers, Chicago, May 16-20, 2004
5. Poggi, M.A., McFarland, A.W., Colton, J.S., and Bottomley, L.A., "A Method for Calculating the Spring Constant of Atomic Force Microscope Cantilevers with a Nonrectangular Cross-section," Analytical Chemistry, Vol. 77, No. 4, 1192-1195 (2005)
6. McFarland, A.W., Poggi, M.A., Bottomley, L.A., and Colton, J.S., "Characterization of Microcantilevers Solely by Frequency Response Acquisition," Journal of Micromechanics and Microengineering, Vol. 15, No. 4, 785-791 (2005)
7. McFarland, A.W., Colton, J.S., Poggi, M.A., and Bottomley, L.A., "Injection-moulded Scanning Force Microscopy Probes," Nanotechnology, Vol. 16, No. 8, 1249-1252 (2005)

8. McFarland, A.W., Poggi, M.A., Doyle, M.J., Bottomley, L.A., and Colton, J.S., "Influence of Surface Stress on the Resonant Behavior of Microcantilevers," *Applied Physics Letters*, Vol. 87, 053505 (2005)
9. McFarland, A.W., and Colton, J.S., "Role of Material Microstructure in Plate Stiffness with Relevance to Microcantilever Sensors," *Journal of Micromechanics and Microengineering*, Vol. 15, No. 5, 1060-1067 (2005)
10. McFarland, A.W., and Colton J.S., "Thermoplastic Polymer Microcantilever Sensors Fabricated via Micromolding," *Journal of Microelectromechanical Systems*, in press (2005)
11. Bottomley, L., Doyle, M., McFarland, Wright, M., Colton, J., and Poggi, M., "Plastic Microcantilever Array Sensors," 207th Meeting of The Electrochemical Society, Quebec City, Canada, May 15-20, 2005
12. D. Cox, A.W. McFarland, S.Y. Liang, and J.S. Colton, "Machining and Molding of Microcantilever Sensors," *Proceedings of the 3rd International Conference on Leading Edge Manufacturing in the 21st Century (LEM 21)*, 865-869, Nagoya, Japan, October 19-22, 2005
13. A.W. McFarland, J.S. Colton, D. Cox, and S.Y. Liang, "Manufacture of Microcantilever Sensors," *Proceedings of the ASME IMECE*, paper 2005-81379, Orlando, November 5-11, 2005