Anal. Chem. **2004,** *76,* 3194−3198

Micromechanical Detection of Proteins Using Aptamer-Based Receptor Molecules

Cagri A. Savran,[†] Scott M. Knudsen,[‡] Andrew D. Ellington,[‡] and Scott R. Manalis*[,†]

Division of Biological Engineering, Media Laboratory, Massachusetts Institute of Technology, 20 Ames Street E15-422, Cambridge, Massachusetts 02139, and Department of Chemistry and Biochemistry, University of Texas at Austin, 2500 Speedway, Austin, Texas 78712

We report label-free protein detection using a microfabricated cantilever-based sensor that is functionalized with DNA aptamers to act as receptor molecules. The sensor utilizes two adjacent cantilevers that constitute a sensor/reference pair and allows direct detection of the differential bending between the two cantilevers. One cantilever is functionalized with aptamers selected for Taq DNA polymerase while the other is blocked with single-stranded DNA. We have found that the polymerase−aptamer binding induces a change in surface stress, which causes a differential cantilever bending that ranges from 3 to 32 nm depending on the ligand concentration. Protein recognition on the sensor surface is specific and has a concentration dependence that is similar to that in solution.

High-throughput proteome analysis has been an important goal since the drafting of the human genome. Although gene chip microarrays can provide information about gene expression, they cannot be directly used to understand protein modification or protein−protein interactions. Numerous studies have been reported on using antibodies as receptors for detecting proteins.[1] Although antibodies can be used to detect proteins with high sensitivity and specificity, they are generally produced in vivo, which introduces difficulties in engineering their properties.

In contrast, aptamers (nucleic acid-binding species), can be selected in vitro and have been produced against a wide range of targets, from small molecules to proteins, to whole cells. Aptamers are DNA or RNA molecules, which can form tertiary structures that recognize and bind to their respective targets. Because nucleic acid backbones are more flexible than their protein counterparts, binding is often accompanied by a structural change that can be utilized for detection of the target, as is done with so-called aptamer beacons.[2] The efficacy of aptamers has been shown on a number of biosensing platforms.[3−7] Furthermore, recent success in the automation of aptamer selection suggests that selection of aptamers on a proteome scale will soon be possible.[8]

Recently, Liss et al. reported label-free detection of IgE using aptamers.[5] They used a quartz crystal microbalance (QCM) system, wherein they functionalized the gold-coated surface of a quartz crystal and observed shifts in its natural frequency upon the mass added by bound antibodies. Another label-free detection method, cantilever-based biosensing, has recently attracted significant attention, primarily due to its scalability. Micromachined cantilevers are batch-fabricated, and due to their small size, arrays can be used in parallel to detect various proteins simultaneously. Researchers have detected proteins by functionalizing cantilever surfaces with various biomolecules including antibodies.[1,9] Binding of target molecules to the immobilized receptor molecules produces a change in surface stress, which bends the cantilever.

The use of aptamers as receptor molecules has not yet been investigated in the context of cantilever-based biosensors. In this paper, we investigate the capability of an aptamer−protein binding event to generate changes in surface stress that bend a flexible micromachined cantilever. We used a receptor−ligand system, which was previously investigated,[10] and characterized in solution. The ligand, i.e., the target molecule, was *Thermus aquaticus* (Taq) DNA polymerase, an enzyme that is frequently used in polymerase chain reaction. The recognition element (receptor) of the sensor was an anti-Taq aptamer modified with a thiol group at one end to enable covalent linking onto a gold surface. The sensor cantilever was functionalized with aptamer molecules, and the reference cantilever was functionalized with oligonucleotides of nonspecific sequence. The differential bending between the two cantilevers was determined directly by using interferometry. We characterize the system in terms of its response to variation in ligand concentration as well as its ability to recognize a particular ligand in a complex mixture and to discriminate against nonspecific binding. Our results indicate that aptamers can be used with

* To whom correspondence should be addressed. Phone: +1 (617) 253 5039. Fax: +1 (617) 253 5102. E-mail: scottm@media.mit.edu.

[†] Massachusetts Institute of Technology.

[‡] University of Texas at Austin.

(1) Wu, G.; Datar, R. H.; Hansen, K. M.; Thundat, T.; Cote, R. J.; Majumdar, A. *Nat. Biotechnol.* **2001**, *19*, 856−860.

(2) Jhaveri, S.; Rajendran, M.; Ellington, A. D. *Nat. Biotechnol.* **2000**, *18*, 1293−1297.

(3) Potyrailo, R. A.; Conrad, R. C.; Ellington, A. D.; Hieftje, G. M. *Anal. Chem.* **1998**, *70*, 3419−3425.

(4) Lee, M.; Walt, D. R. *Anal. Biochem.* **2000**, *282*, 142−146.

(5) Liss, M.; Petersen, B.; Wolf, H.; Prohaska, E. *Anal. Chem.* **2002**, *74*, 4488−4495.

(6) McCauley, T. G.; Hamaguchi, N.; Stanton, M. *Anal. Biochem.* **2003**, *319*, 244−250.

(7) Hwang, J.; Fauzi, H.; Fukuda, K.; Sekiya, S.; Kakiuchi, N.; Shimotohno, K.; Taira, K.; Kusakabe, I.; Nishikawa, S. *Biochem. Biophys. Res. Commun.* **2000**, *279*, 557−562.

(8) Cox, J. C.; Hayhurst, A.; Hesselberth, J.; Bayer, T. S.; Georgiou, G.; Ellington, A. D. *Nucleic Acids Res.* **2002**, *30*, e108.

(9) Arntz, Y.; Seelig, J.; Lang, H.; Zhang, J.; Hunziker, P.; Ramseyer, J.; Meyer, E.; Hegner, M.; Gerber, C. *Nanothechnology* **2003**, *14*, 86−90.

(10) Lin, Y.; Jayasena, S. D. *J. Mol. Biol.* **1997**, *271*, 100−111.

10.1021/ac049859f CCC: $27.50
Published on Web 04/29/2004

cantilever-based sensors for sensitive, specific, and repeatable protein detection.

EXPERIMENTAL SECTION

Nucleic Acids. Nucleic acids were synthesized on an Expedite 8909 nucleic acid synthesis system using reagents from Glen Research (Sterling, VA). Synthesis was performed using standard phosphoramidite chemistry. Following deprotection, DNAs were purified on 8% polyacrylamide/7 M urea gels; bands were excised, eluted overnight at 37 C, and ethanol precipitated. Concentrations were determined based on absorbance at 260 nm. The sequence of the unmodified and modified Taq aptamers are, respectively, 5′ TGGCGGAGCGATCATCTCAGAGCATT-CTTAGCGTTTTGTTCTTGTGTATGA and 5′ S-TTTTTTGGC-GGAGCGATCATCTCAGAGCATTCTTAGCGTTTT-GTTCTTGTGTATGA, where S represents a 5′ C6 thiol linker ($HS(CH_2)_6PO_4$). The sequence of the single-stranded DNA used to block the reference surface is 5′ S-GCGACTGGACATCACGAG. DNA for affinity measurements was radiolabeled at its 5′ end (unmodified Taq aptamer) using T4 polynucleotide kinase and γ-^{32}P-ATP or at its 3′ end (5′-thiol-modified Taq aptamer) using terminal deoxy transferase and α-^{32}P-ddATP. Reactions were performed according to the manufacturer's instructions, DNA was gel purified as above, and recovery was assumed to be 100%.

Affinity Measurements. The binding of DNA aptamers to Taq (Sigma, St. Louis, MO) was measured by nitrocellulose filter binding assay. Radiolabeled aptamer (<0.1 fmol) was combined with varying concentrations of enzyme in a total of 50 μL of binding buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$). Reactions were allowed to come to equilibrium (30 min), and bound species were partitioned from unbound species by passing through successive nitrocellulose and nylon filters under vacuum, followed by washing with 500 μL of binding buffer. Filters were imaged using a Phosphorimager (Sunnyvale, CA), and the fraction bound was calculated using ImageQuant. Values from four such assays were averaged and fit to a standard Langmuir binding isotherm. The experimentally determined affinity was used as an estimate of the concentration of Taq protein in the commercial enzyme using the published K_d for this aptamer.[10]

Complex Protein Mixture. *Escherichia coli* cell lysate was used to mimic a complex mixture. The lysate was formed in 100 μg/mL lysozyme, 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Triton (X-100), 1 mM DTT, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 μg/mL chymostatin. The mass concentration of the lysate stock was 18.5 mg/mL. Lysate solutions were prepared by diluting the stock in Taq binding buffer.

Biosensor and Experimental Setup. The sensor was fabricated from silicon nitride using standard microelectronic fabrication techniques. It has two adjacent cantilevers that form a sensor/reference pair (Figure 1). The relative or differential tip deflection between the two cantilevers is detected directly (as opposed to detecting the two deflection signals separately and subtracting them offline) using interferometry. The cantilever material has a Young's modulus of $E = 180$ GPa[11] and a Poisson's ratio of $\nu = 0.27$.[12] We have previously reported the operation principle, fabrication, and characterization of the sensor.[13] To perform the

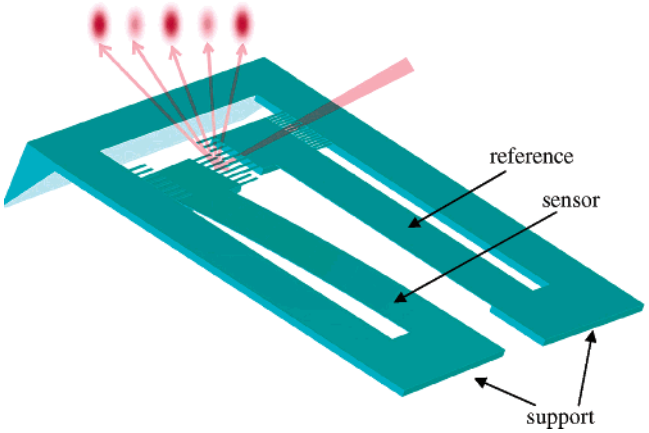

Figure 1. Schematic of the sensor. The sensor and reference cantilevers are supported by L-shaped thick structures that connect them to the die. The die is placed in a stainless steel fluidic chamber. The differential bending is measured directly using interferometry.

experiments reported in this paper, the sensor was placed in a 200-μL stainless steel fluidic cell into which 1-mL samples were injected using an Eppendorf pipet. In the beginning of each experiment, buffer was injected into the chamber and the sensor was equilibrated for a few hours. The optical signal representing differential cantilever deflection was detected using a standard photodetector, converted to voltage using a Keithley 428 current amplifier (with low-pass filter corner frequency ~1 Hz), and acquired with a National Instruments Labview Interface at a sampling rate of 20 Hz.

Functionalization. Before the binding experiment, freshly prepared cantilever surfaces were functionalized with receptor molecules. The sensor was first cleaned using a piranha solution (1:2, H_2O_2/H_2SO_4), washed with H_2O, and dried. Next, 20 nm of Au (with 1-nm Ti as an adhesion layer) was deposited onto one side of the device using an electron beam evaporator. Immediately after the gold deposition, thiol-modified receptor molecules were deposited onto the gold-coated surfaces of the two cantilevers by incubating each cantilever separately (for ~20 min) with a commercially available micropipet that was filled with the appropriate receptor solution. For Taq DNA polymerase detection experiments, one of the cantilevers (sensor) was functionalized with the appropriate aptamer and the other (reference) with single-stranded DNA of nonspecific sequence (ssDNA) (Figure 2a). Both the aptamer and the ssDNA were dissolved in 50 mM TEAA buffer at a concentration of 10 μM. Following functionalization, the sensor was kept at 4 °C overnight. For sequential experiments, cantilever surfaces can be stripped and refunctionalized by removing the Au layer with aqua regia (3:1 HCl/HNO_3) and repeating the above procedure.

RESULTS AND DISCUSSION

Detection Principle. The detection principle is based on surface stress-induced bending of a flexible micromachined cantilever. The relationship between surface stress and cantilever

(11) Barnes, J. R.; Stephenson, R. J.; Woodburn, C. N.; O'Shea, S. J.; Welland, M. E.; Rayment, T.; Gimzewski, J. K.; Gerber, C. *Rev. Sci. Instrum.* **1994**, *65*, 3793−3798.

(12) Senturia, S. D. *Microsystem design*; Kluwer Academic Publishers: Boston, 2001.

(13) Savran, C. A.; Sparks, A. W.; Sihler, J.; Li, J.; Wu, W.-C.; Berlin, D. E.; Burg, T. P.; Fritz, J.; Schmidt, M. A.; Manalis, S. R. *J. Microelectromech. Syst.* **2002**, *11*, 703−708.

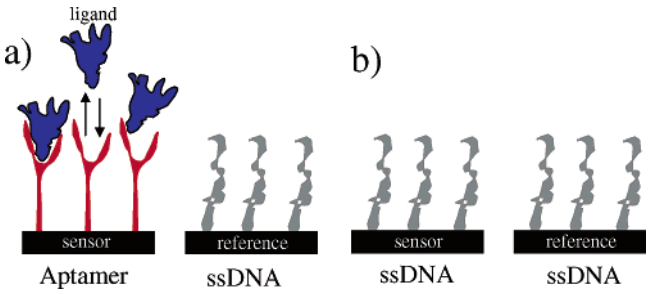

Figure 2. Schematic showing the front view of functionalized sensor/reference cantilevers. Both cantilevers were functionalized on their top (gold-coated) sides. (a) Sensor cantilever has thiol-modified aptamers selected for ligands (Taq DNA polymerase), and reference cantilever has thiol-modified ssDNA. (b) Both cantilevers have thiol-modified ssDNA.

bending is explained by Stoney's equation[14]

$$\Delta z = 3\frac{(1-\nu)}{E}\frac{L^2}{t^2}\Delta\sigma$$

Here, Δz is the cantilever's tip deflection, E and ν are respectively the Young's modulus and the Poisson's ratio of the cantilever material, L and t are respectively the length and the thickness of the cantilever, and $\Delta\sigma$ is the change in surface stress (in units of N/m) on one surface of the cantilever. Adsorption/desorption of molecules on the cantilever surface generates a change in surface stress, which bends the cantilever. As reported by numerous researchers, the deflection of the cantilever is usually measured by focusing a laser beam to the tip of the cantilever and measuring the location of the reflected beam.[1,15] Cantilever-based sensors can be used to detect biomolecules with significant sensitivity. However, a micromachined cantilever is also sensitive to disturbances such as nonspecific adsorption, changes in pH, ionic strength, and especially the temperature[16] of the solution (due to the bimaterial effect caused by a thin metal layer deposited on one side to aid functionalization and laser reflectivity). Therefore, it is advantageous to detect the response of an adjacent reference cantilever and subtract it from that of the cantilever that is used as the sensor. We used a sensor that reveals directly the relative, i.e., differential deflection, between two adjacent identical cantilevers eliminating the need for two separate measurements and their offline subtraction. The two cantilevers form a sensor/reference pair whereby only the sensor cantilever is activated with specific receptor molecules. Hence, the detected signal represents only the receptor−ligand binding that occurs on one cantilever and not on the other. We have previously characterized the sensor's performance and demonstrated the advantage of its differential nature, as well as its application to a model receptor−ligand system.[13,17] In such a device, the reference cantilever can remain unfunctionalized to serve as a control. However, blocking the surface with molecules similar to the receptors helps to reduce nonspecific adsorption[9] to the reference surface.

(14) Stoney, G. *Proc. R. Soc. London* **1909**, *82*, 172−175.
(15) Fritz, J.; Baller, M. K.; Lang, H. P.; Rothuizen, H.; Vettiger, P.; Meyer, E.; Guntherodt, H.; Gerber, C.; Gimzewski, J. K. *Science* **2000**, *288*, 316−318.
(16) Lai, J.; Perazzo, T.; Shi, Z.; Majumdar, A. *Sens. Actuators, A* **1997**, *A58*, 113−119.
(17) Savran, C. A.; Burg, T. P.; Fritz, J.; Manalis, S. R. *Appl. Phys. Lett.* **2003**, *83*, 1659−1661.

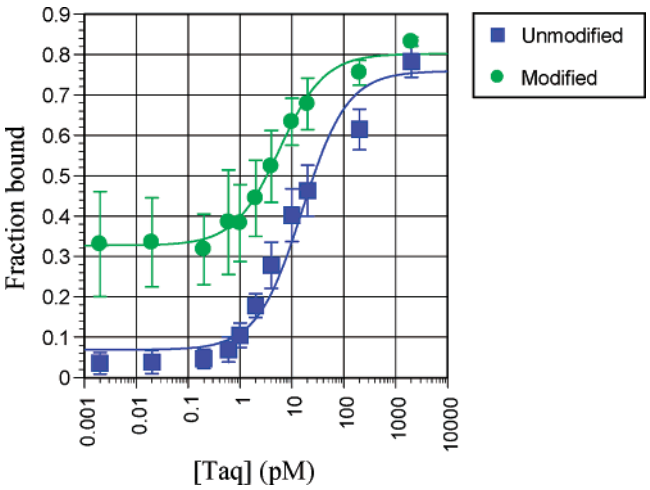

Figure 3. Nitrocellulose filter binding assay of aptamer-Taq binding in solution with both unmodified and thiol-modified aptamers.

Solution Characterization. The aptamer sequence used herein has been previously characterized by Lin and Jayasena[10] (therein referred to as Trnc-21) and is known to bind Taq with a K_d of 9 pM. We have performed aptamer−Taq binding experiments in solution as an estimation of the molar concentration of Taq in the commercially available enzyme and to confirm that modification of the DNA aptamer did not abrogate its binding ability (Figure 3). Nitrocellulose filter binding assays were performed with the unmodified aptamer, and the binding curve was centered on a concentration of 9 pM, corresponding to a concentration of 54 nM for the stock solution; this estimation is used throughout. To attach the aptamer to a gold surface, a modified version was created with a thiol on the 5′ end. Additionally, it has previously been shown that a surface-bound aptamer did not retain its full solution-phase activity,[3] presumably because the protein could not access the entire binding surface for steric reasons. Therefore, we included five additional thymidine residues between the thiol linker and the aptamer sequence to allow additional space between the aptamer and the cantilever surface. A similar set of filter binding assays demonstrated that these changes did not significantly affect the binding ability of the modified aptamer.

Micromechanical Detection. We investigated the specificity of aptamer−protein binding by exposing the sensor (functionalized as in Figure 2a) to two different protein solutions: (1) thrombin, which is a protein that is not expected to interact with the aptamer (although it is known to interact with unrelated DNA sequences); (2) Taq DNA polymerase, which is expected to interact specifically with the aptamer-functionalized cantilever. Figure 4 shows an overlay of the sensor's response to both proteins. We first placed the functionalized sensor in the fluidic chamber, injected buffer into the chamber, and allowed a few hours for equilibration and to establish a stable baseline. Subsequent injection of buffer revealed negligible differential signal and served to verify the stability of the baseline. As expected, injecting a 75 nM thrombin solution revealed no significant differential cantilever bending (Figure 4). We then repeated the experiment, this time by injecting a 500 pM Taq DNA polymerase solution into the fluidic chamber. This resulted in 32 nm of differential cantilever bending, which from Stoney's equation corresponds to 9.6×10^{-3} N/m

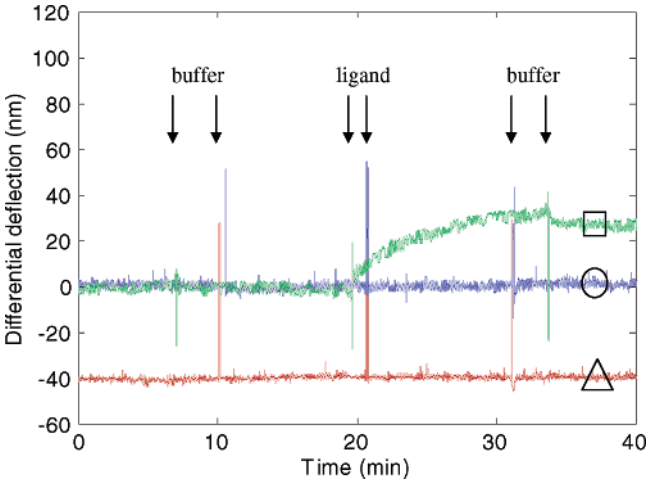

Figure 4. Differential response of aptamer-functionalized sensor to Taq DNA polymerase (□) and thrombin (○) injections. (△) is the response to Taq DNA polymerase when both the sensor and the reference are functionalized with ssDNA (intentionally plotted with a dc offset of −40 nm for clarity).

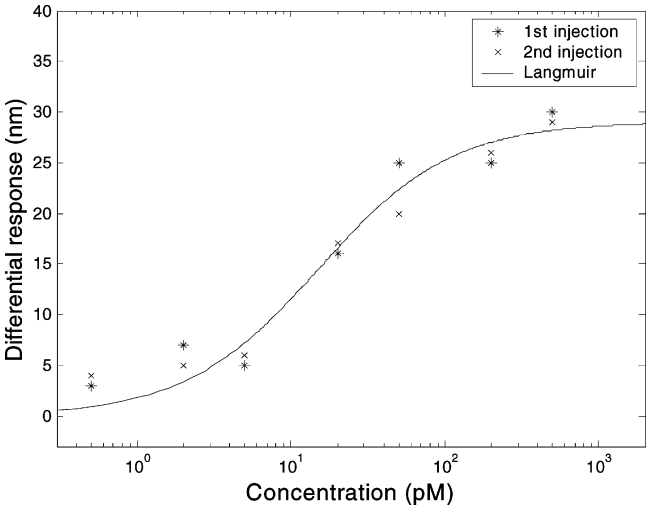

Figure 5. Variation of micromechanical sensor response with Taq DNA polymerase concentration. The experiment was performed twice for each concentration. A Langmuir isotherm fit to the data revealed a K_d of 15 pM.

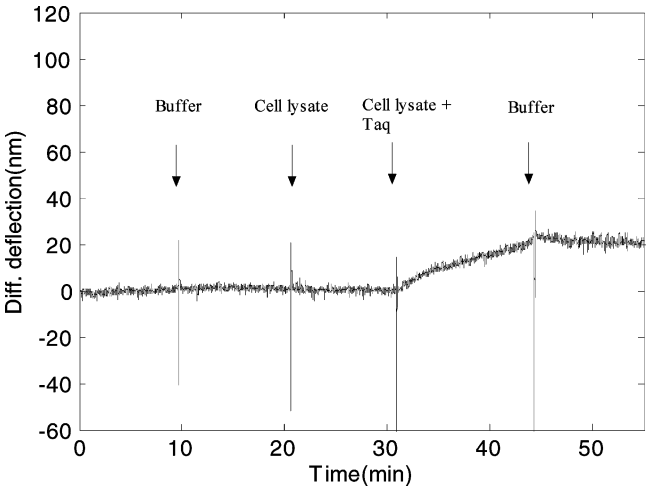

Figure 6. Taq detection in the presence of a complex mixture. Injecting an *E. coli* lysate solution alone did not cause a significant differential response. Injecting the same solution with Taq DNA polymerase generated a clear differential signal.

surface stress. Subsequent buffer injection caused a slight decrease in the signal, possibly because the nonspecifically bound ligands were washed away.

To investigate the differential effectiveness of the device, another experiment was performed in which both the sensor and the reference cantilever were functionalized with ssDNA (Figure 2b). This was performed by stripping the Au layer of the sensor that was previously used for Taq detection experiments, cleaning the device with piranha, depositing a new layer of Au (same as using a new device), and repeating the functionalization process with ssDNA only. Figure 4 also shows the response to Taq DNA polymerase injection, when both the sensor and the reference cantilever are functionalized with ssDNA. As expected, injecting a Taq solution did not cause any differential bending because it does not interact with the ssDNA on either cantilever, or it interacts nonspecifically with each cantilever by the same amount. This response was intentionally plotted with a dc offset of −40 nm for clarity.

The sensitivity of micromechanical detection was investigated by performing binding experiments at a total of seven different Taq concentrations (2 times for each concentration). At the end of each experiment, the reaction was denatured by injecting a 7 M urea solution into the fluidic chamber, and the baseline was reestablished for the new experiment by injecting buffer. Figure 5 shows the differential cantilever bending as a function of Taq concentration (logarithmic scale). The sensor response follows a Langmuir isotherm-type behavior, and the resulting curve can be fit using the least-squares method to reveal a K_d of ∼15 pM. This series of experiments also demonstrates that the functional surface of an aptamer-modified cantilever can be easily regenerated and used for subsequent measurements with acceptable reproducibility.

Finally, we tested the sensor's ability to detect proteins in a complex mixture by using *E. coli* lysate. Again, the device was functionalized with Taq aptamer and ssDNA (for the sensor and the reference cantilevers, respectively), placed in the fluidic cell, and equilibrated in Taq binding buffer. Figure 6 shows that injecting an 18.5 ng/mL cell lysate solution did not reveal a significant amount of differential signal. Injection of the same lysate in combination of 50 pM (4.7 ng/mL) Taq revealed a clear bending signal of ∼20 nm. Thus, the sensor does not respond to a wide variety of biomolecules but maintains the ability to signal the presence of target proteins in a complex mixture (∼4-fold by weight). Furthermore, the signal obtained in this complex background is similar to that obtained under standard binding conditions.

To verify the reproducibility of the binding event on a similar surface, we performed an independent experiment with a QCM system. We used a Maxtek RQCM system with a 9-MHz nominal natural frequency. The gold surface of the crystal was cleaned with piranha and copiously washed with water. The gold surface was then functionalized with the same aptamers used for the cantilever experiments. The crystal was placed in a Teflon fluidic cell, and the whole unit was immersed in a temperature-controlled water bath. Introducing a 50 pM Taq solution into the fluidic chamber resulted in a ∼ 20-Hz reduction in the natural frequency

of the crystal (data not shown), which corresponds to ~7000 Taq molecules/μm^2. This value does not directly apply to the cantilever experiments, since the two surfaces are not exactly the same and differences in surface properties can lead to variations in the number, ordering, and packing of the receptor molecules. However, since both surfaces are gold and were functionalized by the same process, the calculated value can be treated as a rough estimate for the number of Taq molecules that caused cantilever bending. The DNA-based receptor molecules were immobilized in a way similar to that described by Fritz et al.,[15] who reported a surface coverage of ~167 000 DNA probe molecules/μm^2. Assuming a similar coverage by the aptamer receptors, we find a binding efficiency of ~4%.

CONCLUSIONS

We have demonstrated detection of proteins using a micromechanical biosensor that was activated with DNA aptamers. Aptamers were immobilized on cantilever surfaces through a covalent thiol−gold binding, and the aptamer−protein binding generated a change in surface stress that bent a flexible cantilever. We anticipate that cantilever-based biosensors in combination with aptamers will be useful for sensitive and label-free detection of proteins. We also envision that cantilever-based stress sensors will enable understanding certain aspects of aptamers (such as intermolecular forces between aptamers and their effects on conformational changes) that cannot be understood with mass-dependent, label-free detection techniques. As these mechanisms become manifest, we anticipate that analyte-dependent structural rearrangements can be engineered to provide additional sources of surface stress. As a result, this could enable similar detection of small organic analytes that would otherwise yield mass shifts that are too small for label-free detection.

ACKNOWLEDGMENT

This work was supported by National Science Foundation (NSF) Contracts 0243796 (C.A.S., S.R.M.), EIA-0218447, and EIA-0303785 (S.M.K., A.D.E.), Center for Bits and Atoms Contract CCR-0122419, and National Institute of Health (NIH) NIGMS Center for Cell Decision Processes Contracts GM-68762 (C.A.S., S.R.M.), and 8R01EB002043 (S.M.K., A.D.E.). The sensor was fabricated in the MIT Microsystems Technology Laboratories. The cell lysate was prepared by Suzanne Gaudet and John Albeck of MIT Department of Biology.

Received for review January 23, 2004. Accepted March 24, 2004.

AC049859F