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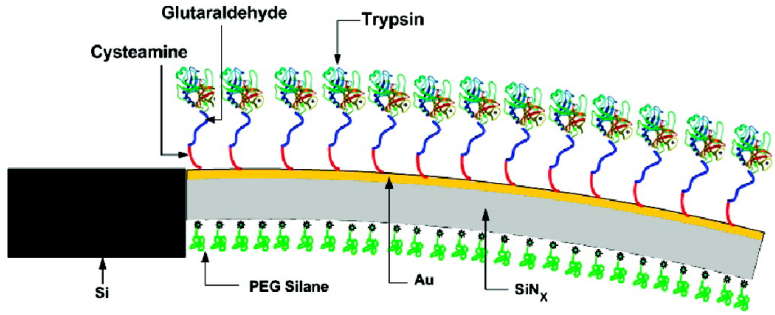

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Quantitative and Label-Free Technique for Measuring Protease Activity and Inhibition using a Microfluidic Cantilever Array

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ABSTRACT

We report the use of a SiN_x based gold coated microcantilever array to quantitatively measure the activity and inhibition of a model protease immobilized on its surface. Trypsin was covalently bound to the gold surface of the microcantilever using a synthetic spacer, and the remaining exposed silicon nitride surface was passivated with silanated polyethylene glycol. The nanoscale cantilever motions induced by trypsin during substrate turnover were quantitatively measured using an optical laser-deflection technique. These microcantilever deflections directly correlated with the degree of protease turnover of excess synthetic fibronectin substrate ($K_M = 0.58 \times 10^{-6}$ M). Inhibition of surface-immobilized trypsin by soybean trypsin inhibitor (SBTI) was also observed using this system.

Introduction. Enzyme−substrate interactions are vital in many biological functions, and aberrations in specific enzyme reactions can often lead to disease. The insight gained from studies that probe these interactions have been important to understand their biological roles and have also helped to identify biomarkers as well as therapeutic targets.[1] Clinical diagnostics often relies on quantifying such biomarkers. However, as exemplified by the use of prostate specific antigen (PSA) in prostate cancer diagnosis, mere quantification can lead to high rates of false positives and negatives, making it inadequate for specificity in diagnosis.[2,3] It has been proposed that the activity of protein enzymes, particularly those implicated in disease, would be a better indicator of disease states while simultaneously serving as therapeutic targets.[4] This warrants deeper exploration and development of quantitative techniques that can measure the activity of disease-related enzymes.

Proteases account for approximately 2% of all proteins in human beings and have been shown to play important roles in various physiological processes including bioregulation and immune response.[5] Due to their ubiquitous presence in the human body, the dysregulation of protease activity commonly results in a variety of diseases including hemophelia, cancer, and heart disease. Their activity and catalytic turnover of substrate make them an ideal biomarker for the detection and intervention of disease. Several current therapeutics are aimed at inhibiting upregulated proteases. This presents an obvious need for a technique that can readily monitor protease−substrate interactions and screen for inhibitors of proteases.[6]

Various methods such as protein microarrays[7−10] and immunosorbent assays[11−13] are commonly used for biomarker detection in both clinical applications and in research. However, such approaches rely on the expensive and time-consuming techniques of radio and/or fluorescent/chemiluminescent labeling of the samples. More importantly, these techniques only detect the presence of a specific protein and commonly do not discriminate between active and inactive (a.k.a. zymogen) forms. Other quantitative and label-free platforms include the measurement of changes in optical constants by surface plasmon resonance (SPR),[14,15] changes in mass using quartz crystal microbalances,[16−18] changes in refractive index using resonance mirrors,[19,20] changes in surface stress through the use of microcantilevers,[21] and electrophoretic separation.[22,23]

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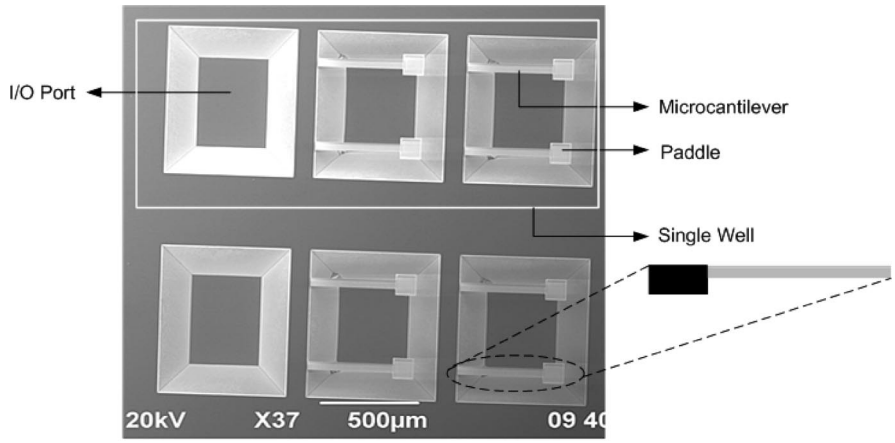

Figure 1. SEM picture of two microcantilever wells. Each cantilever is 400 μm long, 40 μm wide, and 0.5 μm thick in size.

Microcantilever arrays are an attractive label-free option because of the use of low-cost silicon microfabrication, the ability to monitor multiple reactions in real time, high sensitivity to biological interactions due to their small size, and the use of small sample volumes.[24] Microcantilevers are nanomechanical transducers that convert intermolecular reaction forces into measurable cantilever deflection.[25] When specific interactions among biomolecules occur on one surface of a microcantilever, the cantilever experiences a change in surface stress as a result of the reduction in free energy. This biomolecular interaction is translated into the bending of the microcantilever.[21,24,26−30] Numerous studies have reported the use of microcantilevers as high throughput quantitative biosensors for the detection of hybridization of DNA,[26,29] DNA aptamer-protein interaction,[28] antibody−antigen interactions,[24] melting of DNA.[25]

In this study, we demonstrate for the first time the application of a two-dimensional (2D) microcantilever array to quantitatively detect substrate cleavage by a model protease that is immobilized to its gold surface. This was achieved via a spacer terminated with reactive aldehyde and primary amine functional groups. The high sensitivity and quantitative response of the microcantilever to the protease upon introduction of a synthetic fibronectin substrate allowed for the calculation of the Michaelis−Menten constant, which was found to be an order of magnitude lower than the value measured in solution as expected.[31] The similarity between the surface and the solution measurements quantitatively demonstrates that the technique used to immobilize the protease to the surface of the microcantilever minimally affects the protease's activity. We demonstrate the ability of this microcantilever array to serve as a platform for quantitatively screening inhibitors of known proteases. Furthermore, label-free measurement of the concentration of a given protease substrate can be achieved upon calibration of the microcantilever system.

Experimental Set-Up and Procedure. *2D Cantilever Array and Optomechanical Readout*. Figure 1 shows the scanning electron microscope (SEM) image of the 2D cantilever array. The fabrication process for the microcantilever array chip and the design of optical multiplexing are described in detail in a previous report.[32,33] The array contains individually addressable reaction wells, each of which contains multiple (4−6) cantilever sensors made of low stress 0.5 μm thick SiN_x film, which was patterned to define the cantilevers. Then, a 25 nm thick gold film was thermally evaporated on the wafer with a 5 nm thick layer of chrome, which acts as an adhesive between the gold and the silicon nitride films. The gold film was patterned, and the bulk wet etching of silicon was carried out using a potassium hydroxide solution. The microcantilevers were then released in a critical point dryer. The response of the multiple (4−6) cantilevers in each chamber can be averaged to increase the accuracy of the measurements. Each cantilever has a rigid paddle structure at its end to provide a flat reflecting surface for monitoring the changes in deflection using optical techniques.

In order to simultaneously image multiple wells, it is necessary to construct a ray optics-based whole field illumination system. Briefly, a laser beam is expanded and reflected off the cantilever array chip. The flat paddles at the end of the cantilevers produced spots on a CCD camera. By quantitatively measuring the motion of each spot, it is possible to detect the deflection of cantilever beams in a fashion similar to an atomic force microscopy system. The distance between the charge coupled device (CCD) and the cantilever array chip is directly proportional to the movement of the spot on a CCD screen and inversely proportional to the intensity of the spot. The shorter the distance, the closer the cantilever spots are to each other on the CCD screen allowing more spots to be imaged by the CCD.

Microcantilevers were calibrated by making use of the thermal bimorph effect between adjacent gold and silicon nitride layers. The thermo mechanical sensitivity of 208 $\pm$ 14 nm/K for the 200 μm long cantilevers was observed, and the surface stress sensitivity was estimated to be 24.5 $\pm$ 1.65 $mJ/m^2 \cdot K$ (ref 32).

Cantilever Preparation. Microfabricated Au/SiN_x cantilevers were cleaned in acetone and then in isopropanol, followed by a deionized water (DI) wash prior to the experiment. The chip was then equilibrated in isopropanol followed by coating of polyethylene glycol (PEG) silane (2-[methoxy(polyethylenoxy)propyl] trimethoxysilane, Sigma Aldrich) on the nitride surface using a silane protocol

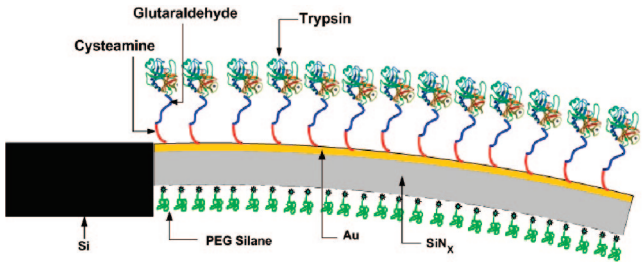

Figure 2. Conceptual diagram of the cantilever with the functionalized biomolecules. The gold side of the cantilever was coated with cysteamine and gluteraldehyde. Trypsin was covalently attached to the aldehyde group at the free end of gluteraldehyde. The silicon nitride side of the cantilever was coated with PEG silane.

described in Papra et al. and Yue et al.[34,35] This technique has been shown to prevent the nonspecific adsorption of proteins on the nitride surface of the cantilever.

The cantilever array chip was washed with DI water using a pipet. Cysteamine (5 mM, $HS-C_2H_4-NH_2$, Sigma Aldrich) was dissolved in phosphate buffer saline (PBS; 1×, pH 7.2, Invitrogen) and injected into the multiple wells. The cantilevers were soaked in this solution for 3 h to form a self-assembled monolayer on the gold surface of the cantilevers via gold-thiol chemistry. Gluteraldehyde (2.5% w/w in 1 × PBS, $OHC-C_3H_6-CHO$, Fluka) was added to the well, forming an amide bond with the immobilized cysteamine. The chip was then incubated for 1 h to immobilize the tryspin (Gibco) via a cross-reaction between the primary amines with the aldehyde group of gluteraldehyde.[36] This immobilization procedure is illustrated in Figure 2. The chip was washed multiple times with PBS to remove any unconjugated trypsin. A PBS solution containing lysine (1 M in 1 × PBS, Sigma Aldrich) was then introduced to react with any remaining aldehyde groups of gluteraldehyde.

Quantitatively Measuring Microcantilever Deflection. To evaluate whether the deflection of the microcantilevers was solely due to the activity of trypsin, the results of the microcantilevers coated with the immobilized trypsin with those from a control experiment, where the surfaces were conjugated with the spacer but lacked the protease, were compared. For control experiments, the gold surface of the cantilever was first immobilized with cysteamine and incubated with gluteraldehyde. The reactive gluteraldehyde groups were then prevented from further reaction by injecting a lysine solution, which "capped" the end of the spacer via reaction with the aldehyde groups. As illustrated in Figure 3a, the end product of these reactions was a cantilever surface that was passivated by a similar spacer as those in the trypsin experiments but without the protease. Following these immobilization reactions, a commercially available trypsin substrate which mimics the natural fibronectin substrate (1 × PBS, 1 mg/ml, amino acid sequence: 1377−1388, Sigma Aldrich) was directly dissolved in 1 × PBS and introduced to the cantilever. The deflection of cantilevers was observed in real time.

To evaluate the ability to screen for protease inhibitors, trypsin was immobilized using the same cysteamine-gluteraldehyde chemistry as described previously. The unbound reactive gluteraldehyde group was then quenched with lysine solution. A solution containing soybean trypsin inhibitor (SBTI, Sigma Aldrich) was introduced to the cantilever as shown in Figure 3b, and the response was observed in real time. A solution containing the fibronectin substrate was then injected in the well, and the deflection, if at all, was observed in real time.

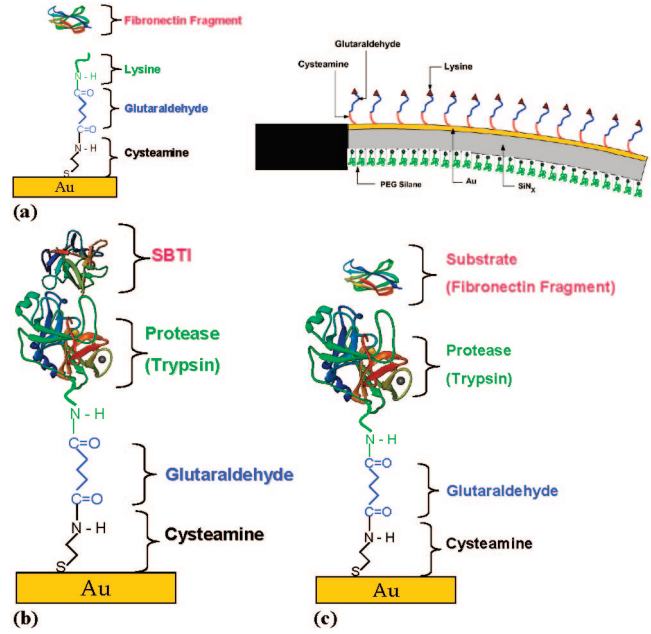

Figure 3. (a) Illustration of microcantilever immobilized for a control experiment in the absence of protease. Cantilevers were functionalized with cysteamine and gluteraldehyde. Aldehyde groups of gluteraldehyde were quenched with 1 M Lysine solution. Then, the substrate (fibronectin fragment) solution was injected into the well. (b) Schematic of Au surface of microcantilevers functionalized with Trypsin. Cantilevers were functionalized with cysteamine and gluteraldehyde. Trypsin was immobilized using aldehyde-primary amine chemistry. The inhibitor was then injected into the well. (c) Visualization of Au surface of microcantilevers immobilized with protease. Cantilevers were functionalized with cysteamine and gluteraldehyde. Trypsin was immobilized using aldehyde-primary amine chemistry. The substrate was then injected into the well.

The sensitivity of the microcantilever to quantitatively measure substrate turnover by a protease was then evaluated. The response of the immobilized trypsin against various concentrations of the fibronectin substrate was analyzed. By using the same protocol as the inhibition experiments but in the absence of any SBTI, the response of the cantilever versus a wide range of substrate concentrations was measured. The protocol used is illustrated in Figure 3c. The deflection of microcantilevers was recorded as a function of substrate concentration using the optical detection system.

Solution Comparison Measurements. To study whether the physical immobilization of a protease onto the surface of a cantilever interferes with its ability to turnover substrate, enzyme kinetic studies of trypsin and a similar substrate were carried out in solution. The goal of these studies was to compare the Michaelis−Menten constant of the solution measurements with those calculated for the immobilized

trypsin. Enzyme kinetics were measured by tracking the activity of trypsin against a synthetic mimic of fibronectin that has been derivatized with a 7-amino-4-carbamoylmethylcoumarin (ACC) fluorophore as described previously.[37] Upon hydrolysis of the ACC-peptide bond by the protease, the fluorescent properties of ACC change so that it becomes fluorescent at 460 nm with excitation at 380 nm. The fibronectin substrate used for the microcantilever experiments had the following sequence: H_2N-His-Ser-Arg-Asn-Ser-Ile-Thr-Leu-Thr-Asn-Leu-Thr-COOH, and the synthetic substrate was synthesized with the following sequence: Ac-His-Ser-Arg-ACC (Ac = acetylated N-terminus). The shorter sequence was chosen to minimize the interference caused by the other basic residues that may also react with trypsin and confound an accurate measurement of the turnover using ACC as the fluorophore. The peptide and fluorophore were synthesized and purified following a procedure that has been previously described.[38]

The substrate was diluted at various concentrations (1.3×10^{-5} to 6.5×10^{-7} M) into a 96 well fluorescent plate (Dynex Technologies, Chantilly, VA) in a buffer containing 50 nM TRIS (pH 8.1), 150 nM NaCl, and 1 mM $CaCl_2$. Trypsin (5 nM) was dissolved in a similar buffered solution and added to the substrate containing wells. Reaction rates were immediately monitored at 25 °C by following the increase in fluorescence (SpectraMax Gemini EM). The kinetic parameters under these conditions were determined by fitting the data to the Michaelis−Menten equation using the curve-fitting program Kaleidagraph (Synergy Software) and calculated to be $K_m = 6.7 \pm 0.9 \times 10^{-6}$ M, $k_{cat} = 8.4 \pm 0.9 \times 10^{-6}$ s^{-1}.

Results and Discussion. When the fibronectin fragment substrate is introduced to the reaction chamber and allowed to react with the immobilized trypsin, the cantilever is observed to undergo a downward deflection with the Au film on top, as shown in Figure 4a. The cantilever deflection reaches a steady state within about 15−30 min. It was observed that the magnitude of the steady-state deflection increases as the fibronectin fragment concentration is increased. The deflection of microcantilevers is caused by a change in the surface stress in the gold layer of the cantilever. The relationship between surface stress and cantilever bending follows Stoney's formula[39]

$$\Delta z = \frac{3 \cdot \Delta\gamma \cdot (1 - \upsilon_1) L^2}{E_1 t_1^2} \quad (1)$$

where Δz is the cantilever's tip deflection, $\Delta\gamma$ is the change in surface stress, υ_1 is the Poisson's ratio of the thick layer, E_1 is the Young's modulus of the thick layer, t_1 is the thickness of the thick layer, and L is the length of the cantilever.

Figure 4b shows a plot of the change in surface stress as a function of substrate concentration for the case where trypsin was immobilized on the gold surface of microcantilevers. These data points were fitted with the Langmuir adsorption isotherm model.[8,27,40−42] This model can be used to derive the surface−solution equilibrium constant K assuming that a ligand (substrate) striking the receptor

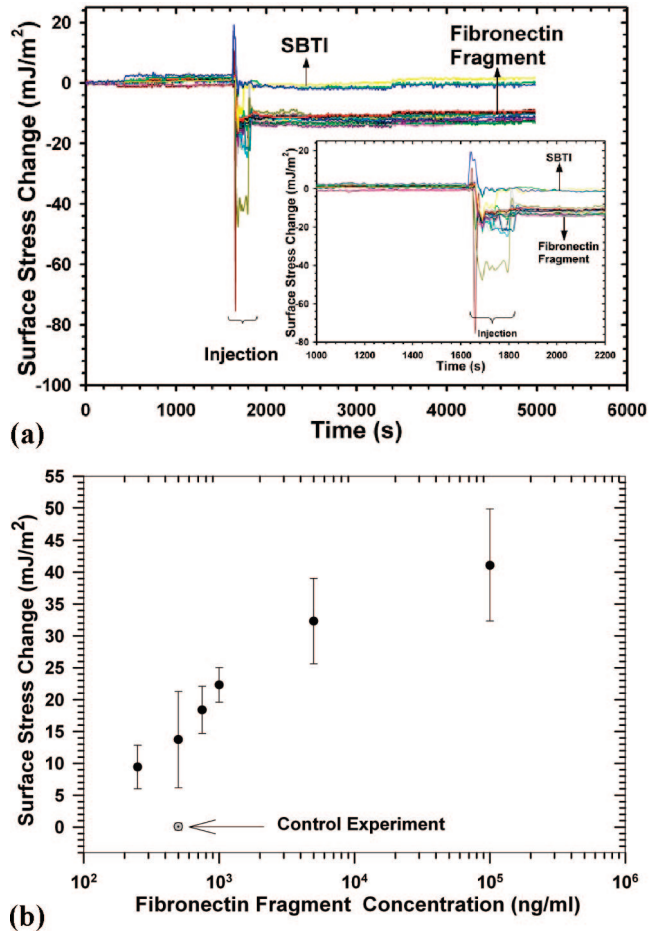

Figure 4. (a) Response of microcantilevers upon injection of the substrate (fibronectin fragment at 1 μg/ml) and SBTI. Real time deflection data for each cantilever was represented in a different color. (Inset) Zoomed in view of the deflection of microcantilevers just before and after the injection of biomolecules. (b) Surface stress change (signal) vs concentration of substrate plot for fibronectin fragment. This plot shows the typical Langmuir isotherm behavior with the saturation of signal for higher concentration. In all of the experiments, microcantilevers were functionalized with cysteamine and gluteraldehyde. Trypsin was immobilized using aldehyde-primary amine chemistry. In the case of the control experiment, cantilevers were functionalized with cysteamine and gluteraldehyde. Aldehyde groups of gluteraldehyde were quenched with 1 M Lysine solution. Then, the substrate (fibronectin fragment) solution was injected into the well. The real time data has been shown in Figure 7.

(protease) that is already occupied during the cleavage reaction does not adsorb and that the receptor−ligand binding events are independent of each other.[27,41]

Accordingly, the fractional surface coverage (θ) is related to the concentration of the ligand in the solution (c) and the binding constant (K) by the following relation

$$\theta = \frac{cK}{1 + cK} \quad (2)$$

The fractional coverage (θ) determines the number of binding events which in turn determines the nanomechanical cantilever bending signal. Hence, we assume that the change in surface stress ($\Delta\gamma$) is proportional to the fractional surface coverage (θ) such that

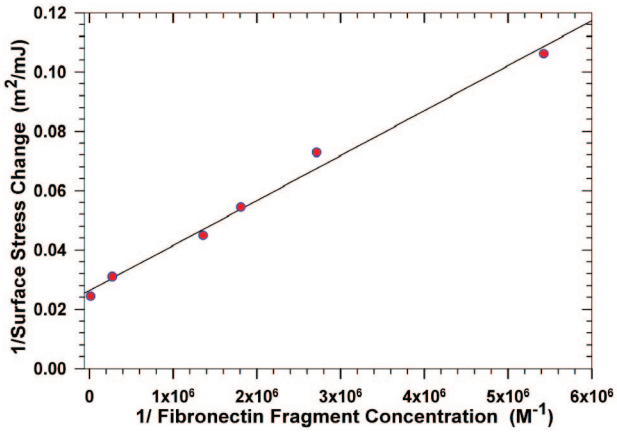

Figure 5. (1/Surface stress change) vs (1/concentration of fibronectin fragment) plot for trypsin-fibronectin fragment interaction. The Michaelis−Menten constant thus derived from the plot is $K_M = 0.58 \times 10^{-6}$ M.

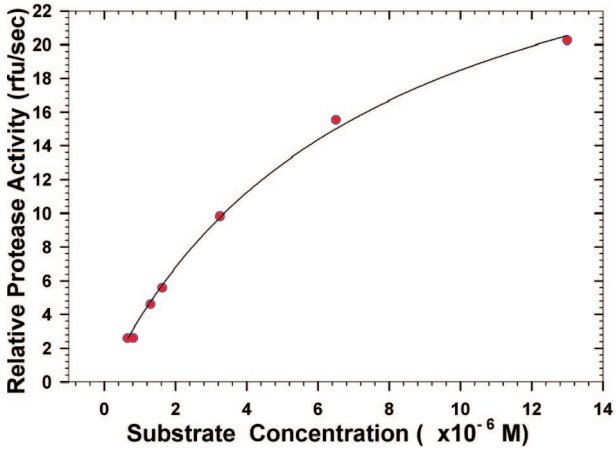

Figure 6. Example of kinetics plot used to determine the solution phase turnover of 5 nM trypsin vs Ac-His-Ser-Arg-ACC ([substrate] $= 1.3 \times 10^{-5} - 6.5 \times 10^{-7}$ M) in 50 mM TRIS (ph 8.1), 150 mM NaCl, and 1 mM $CaCl_2$. The Michaelis−Menten constant found from the plot is $k_M = 6.7 \pm 0.9 \times 10^{-6}$ M.

$$\Delta\gamma = a^* \frac{K \times c}{1 + K \times c} \tag{3}$$

where a^* is a constant for given cantilever geometry.

Figure 5 shows a plot of $\Delta\gamma$ versus $cK/(1 + cK)$ describing the substrate−trypsin interaction. Since this is a reaction between a protease and its substrate, the reaction constant is same as the Michaelis−Menten constant for the protease in solution. As expected, the data can be fit to a straight line, which further substantiates the Langmuir isotherm model. From the slope and intercept of the plot, the equilibrium constant K can be extracted. For the case of immobilized trypsin and fibronectin fragment, the equilibrium constant was $K_M = 0.58 \times 10^{-6}$ M. It is generally observed that the surface−solution equilibrium constant is lower than the equilibrium constant in solution.[31] Hence, it compares well with that obtained from the experiments carried out with trypsin and fibronectin fragment in solution ($K_m = 6.7 \times 10^{-6}$ M) as shown in Figure 6. As expected, the reaction constant in solid−solution medium is lower than that observed in solution. One possible explanation for the lower value of the immobilized trypsin is due to the reduction in the total degrees of freedom available to the protease, limiting the flexibility in the types of interactions with its substrate, compared with it in solution. Also, trypsin molecules can get immobilized in all possible random orientations on the Au surface of microcantilever thus effectively reducing the number of molecules that can interact with the substrate. It should also be noted that ACC is a better leaving group that facilitates the cleavage of the substrate relative to the peptide bond in the fibronectin fragment.

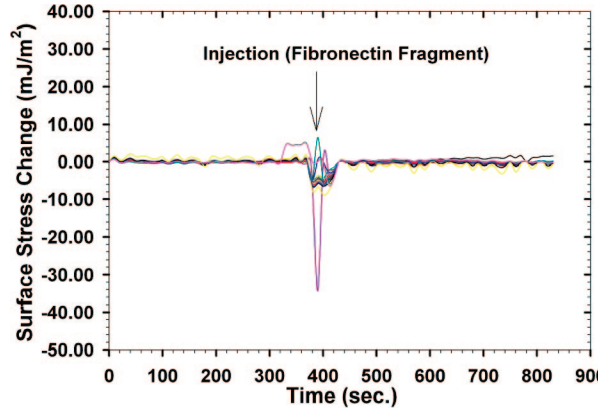

Figure 7. Insignificant deflection of microcantilevers upon injection of the substrate (fibronectin fragment) in a control experiment. Cantilevers were functionalized with cysteamine and gluteraldehyde. Aldehyde groups of gluteraldehyde were quenched with 1 M lysine solution. Then, the substrate (fibronectin fragment) solution was injected into the well.

In the control experiment, the injection of fibronectin fragment solution in the absence of trypsin on the gold surface leads to no significant deflection of microcantilevers as shown in Figure 7. This provides further evidence that the signal observed in the prior experiments with the immobilized trypsin is due to the interaction of the fibronectin substrate with the protease, ruling out the possibility that the observations were due to a direct reaction between the substrate and the surface.

To evaluate whether the microcantilever array can be used as a platform for screening for inhibitors of a protease, we introduced a known trypsin inhibitor to the immobilized protease, prior to the addition of the substrate. The first experiment was to see if there would be a response of the cantilever to the inhibitor alone, in the absence of substrate. The injection of SBTI did not lead to any observable microcantilever deflection, as shown in Figure 8 as well as in Figure 4a. It has been proposed that the degree of conformational change when a protease interacts directly with an inhibitor is small;[43] thus, we expected very minimal surface stress change when we introduced SBTI to the immobilized trypsin. As observed under these conditions, the microcantilevers did not undergo any substantial deflection, particularly when compared with the motions observed when substrate is introduced to the protease in the absence of any inhibitor.

The second experiment investigated whether the inhibition of trypsin by SBTI could be observed using our microcantilever array. As with the other experiments, we did not

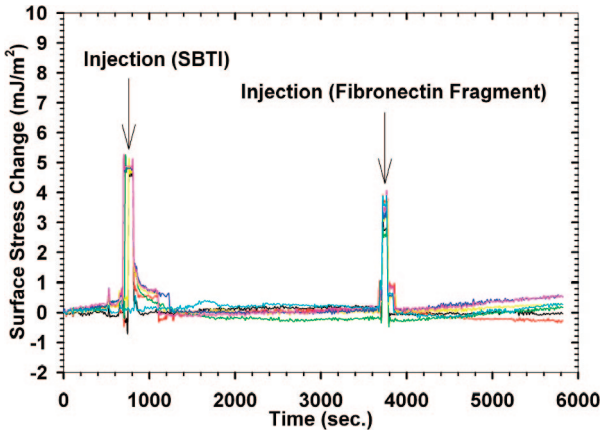

Figure 8. Insignificant deflection of microcantilevers upon injection of the inhibitor (SBTI) and subsequent injection of substrate (500 μg/ml, fibronectin fragment) in the same wells of the chip. Microcantilevers were functionalized with cysteamine and gluteraldehyde. Trypsin was then immobilized using aldehyde-primary amine chemistry.

observe any significant deflection of the microcantilevers upon injection of inhibitor prior to the addition of substrate. The subsequent injection of the fibronectin substrate did not result in any observed deflections, unlike the experiments that were conducted in the absence of SBTI. From these results, we conclude that the immobilized trypsin is indeed inhibited by SBTI under our conditions and unable to cleave the substrate.

Conclusion. We have shown the novel use of a microcantilever array to quantitatively measure the activity of a model protease, trypsin. We demonstrate that its immobilization onto a microcantilever surface minimally affects its ability to turnover substrate. We were also able to quantitatively measure the nanomechanical response of the cantilever to an interaction between a protease and a substrate (trypsin-fibronectin fragment) and, more importantly, detect its inhibition (using SBTI and substrate). From the generation of surface stress on a cantilever surface, it was inferred that the protease undergoes a substantial conformational change on the surface when it interacts with the substrate as compared with the inhibitor.

We showed that the mechanical signals of cantilevers resulting from protease−substrate interactions followed the Langmuir isotherms, which can be translated to measure substrate turnover of a given protease−substrate interaction. In the case of the trypsin−fibronectin interaction, the Michaelis−Menten constant, K_M, was found to be 0.58×10^{-6} M which matched well within the order of magnitude to that measured in solution. It should be noted that the reaction constant for the surface−solution interaction was found to be 1 order of magnitude lower than that in solution possibly because of reduced degrees of freedom. Thus, the microcantilever array can be a valuable tool to measure protease−substrate interactions and to calculate the Michaelis−Menten constant in a label free manner for a given protease−substrate pair. In the process, we have also shown that the microcantilever system can be calibrated for the given protease−substrate pair. And this curve can then be used to measure the unknown concentration of a label-free substrate solution by simply introducing the solution to a well containing the functionalized microcantilevers. These results demonstrate the potential use of a microcantilever array, which has been functionalized with a given protease, as a biosensor.

These results also provide a proof of principle, demonstrating that a microcantilever array can serve as a platform to quickly screen for inhibitors of known proteases. We have demonstrated that the response of the cantilever quantitatively reflects the interaction of an immobilized protease in the presence of substrate, and we have also demonstrated its lack of substrate turnover when the protease is inhibited. The use of an optical reflection technique to monitor cantilever deflection is amenable for monitoring several reactions at the same time in a multiplexed format. This is ideal for quickly screening and identifying which proteases are targeted by a known inhibitor by immobilizing different proteases on individual cantilevers in an array format. In addition, since the protease is immobilized, solutions containing inhibitors can be quickly replaced allowing for the rapid screening of a panel of known inhibitors against a single immobilized protease. The quantitative measurements can then be used to understand the degree of inhibition and identify the most potent and specific inhibitors. We are focusing our current investigations into optimizing the ability of our array to identify these inhibitors against known disease-associated proteases, as a potential novel method for designing specific therapeutics.

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