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Label-Free Protein Recognition Two-Dimensional Array Using Nanomechanical Sensors

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ABSTRACT

We demonstrate two-dimensional multiplexed real-time, label-free antibody–antigen binding assays by optically detecting nanoscale motions of two-dimensional arrays of microcantilever beams. Prostate specific antigen (PSA) was assayed using antibodies covalently bound to one surface of the cantilevers by two different surface chemistries, while the nonreaction surfaces were passivated by poly(ethylene glycol)-silane. PSA as low as 1 ng/mL was detected while 2 mg/μl of bovine serum albumin induced only negligible deflection on the cantilevers.

Microcantilevers are nanomechanical transducers, which convert intermolecular reaction forces to detectable cantilever deflection in nanometers.[1−9] When specific reactions among biomolecules occur on one surface of a cantilever, the reactions induce a change in the surface stress as a result of the free energy reduction. The cantilever deflects due to the change of surface stress and indicates the occurrence of the specific biomolecular reaction. We and other researchers have previously demonstrated DNA and protein detection with one,[2−4] a couple,[7,8] or at most eight cantilevers in a one-dimensional (1D) array.[1,5,6,9]

Protein markers have been under intensive studies as an important class of "molecular markers" of cancers, along with other diseases. Stamey and his colleagues first reported that serum levels of prostate specific antigen (PSA) were correlated with increasing palpable stages of prostate cancer.[10] Research has later shown that cancer is a sufficiently complex disease that it cannot be diagnosed or predicted by a single protein marker. Instead, a hypothesis that has been partially successful and is gaining wider acceptance is that a "profile" of many protein markers may be more relevant for reliable cancer diagnosis. With ovarian cancer as an example, instead of using only CA-125 as a single biomarker, incorporating additional biomarkers such as IL-8 resulted in increased classification specificity.[11] Thus, a multiplexed platform capable of screening many possible protein markers at a time is a highly desirable solution for determining the "diagnostic profiles" of various cancers. We recently reported DNA hybridization studies using two-dimensional (2D) microcantilever arrays, which are truly scalable to hundreds of simultaneous reactions.[12−14] In this study, for the first time we demonstrate a label-free immunoassay with a 2D microcantilever array.

The 2D cantilever array chip contained 80−120 reaction wells, where each well consisted of a microfluidic chamber containing 4−8 independent cantilever sensors. All cantilevers within a given chamber were necessarily exposed to the same test solutions. These silicon nitride cantilevers were fabricated on a silicon chip, which was then bonded to a glass chip containing the reaction wells[12,13] (Figure 1). The cantilevers were 0.5 μm thick, 40 μm wide, and 200−400 μm long. They were coated on one surface with a 25 nm gold layer, which served as the surface for immobilizing capture molecules (antibodies). A collimated laser light beam with an expanded spot size about the same size as the whole cantilever array (~2.5 cm in diameter) illuminated the gold surface of the cantilevers from the glass side. The laser light reflected off each cantilever's end pad and was collected as an array of "spots" by a charge coupled device (CCD) camera. Thus, each cantilever's deflection was monitored by tracking its corresponding light spot on the CCD

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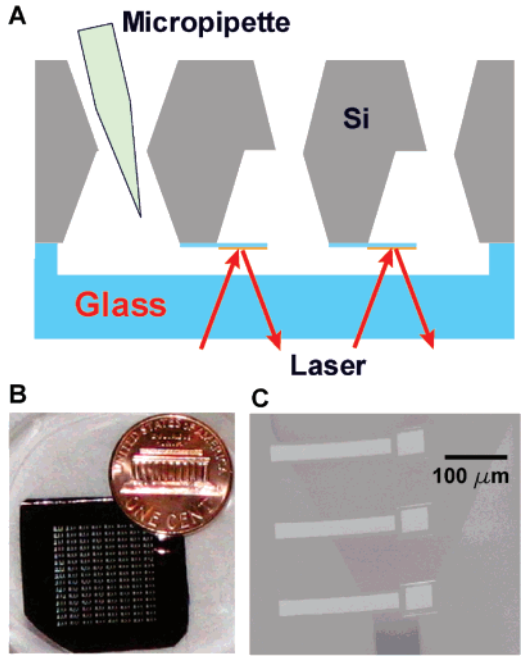

Figure 1. Two-dimensional microcantilever array chip. (a) Schematic of a reaction well. There were multiple cantilevers in each reaction well. Laser light reflected off a cantilever's end pad was used to monitor the deflection of cantilevers. (b) A chip soaked in DI water. (c) A scanning electron micrograph of 3 cantilevers in a reaction well.

camera.[12,13] Greater detail of the parallel detection can be found in the Supporting Information.

To achieve sensitive and repeatable performance of microcantilever sensors for the detection of protein−protein interactions, we explored two different surface chemistries for passivation of inactive surfaces and three different surface chemistries for functionalization of active surfaces with probe molecules (antibodies). The functionalization is critical to the cantilever sensing because it determines the surface density of receptor molecules and thus the amount of the binding events on the active surface. It may also affect how efficient the transduction is from the biomolecular interactions to the surface stress change on the cantilevers. Additionally, an effective passivation can significantly reduce the nonspecific binding so that the background signal can be minimized. Figure 2 illustrates four combinations of passivation and functionalization chemistries, which we examined.

2-[Methoxypoly(ethyleneoxy)propyl]thimethoxysilane, a silane conjugated polyethylene-glycol chain (henceforth referred as PEG-silane) and bovine serum albumin (BSA) were used as two alternatives for passivation of the silicon-nitride surface of the cantilevers and inner surfaces of the fluidic chambers. PEG-silane was grafted to the surfaces using a protocol similar to that described by Papra et al.[15] Briefly, the microcantilever chips were immersed in a freshly prepared solution of PEG-silane in toluene for passivation, followed by rinsing with toluene, ethanol, and deionized (DI) water, sequentially. Alternatively, when BSA was used for passivation, the chips were immersed in a solution of 2 mg/mL BSA in phosphate buffered saline (PBS) solution overnight at 4 °C. The chips were then rinsed thoroughly

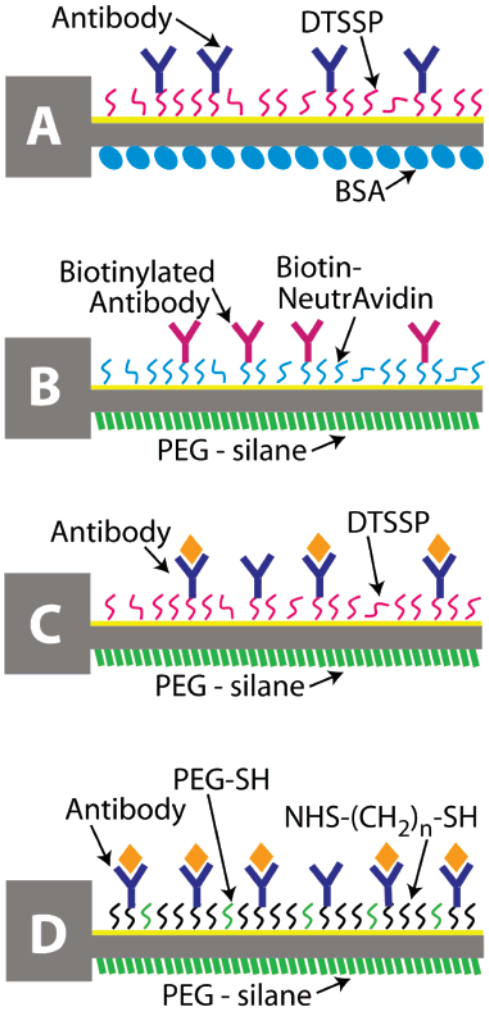

Figure 2. We examined the cantilever response to antigen−antibody interactions using four surface chemistry combinations including (A) DTSSP linkage (functionalization) and BSA (passivation); (B) Biotin−NeutrAvidin affinity (functionalization) and PEG-silane (passivation); (C) DTSSP linkage (functionalization) and PEG-silane (passivation); (D) Mixed SAM linkage (functionalization) and PEG-silane (passivation).

with DI water and were ready for the subsequent surface functionalization.

For the gold-coated surface of the cantilever, we explored three methods for immobilization of antibodies (functionalization). The first immobilization method utilized 3,3′-dithiobis-[sulfosuccinimidylpropionate], DTSSP, to create a direct covalent link between the antibody and the gold surface.[2] DTSSP was immobilized onto the gold surface of a cantilever in citrate buffer. Anti-PSA antibody could covalently link to the DTSSP on the surface, through the reaction between its NHS groups and the amine groups of the antibody. The second method of surface functionalization was to form a mixed self-assembled monolayer (SAM) using a mixture of NHS (*N*-hydroxysuccinimide) thiol (HS-$[CH_2]$-11-$[OCH_2]_6$-COO-NHS), henceforth referred as NHS-thiol, and PEG-thiol (HS-$[CH_2]_{11}$-$[OCH_2]_6$-OH). It is known that DTSSP is unlikely to form a highly ordered monolayer for protein immobilization. Herrwerth et al. developed a covalent method of coupling protein molecules to gold surfaces by

using a SAM of carboxy-functionalized PEG alkanethiol.[16] Frederix et al. demonstrated that mixed SAMs provide enhanced performance as affinity biosensor interfaces.[17] We used a mix ratio of 4:1 between NHS-thiol and PEG-thiol to form the SAM on the gold surface of a cantilever. The antibody was also covalently bonded to the crosslinker with the covalent reaction between the NHS ester and antibody's amine groups. The third means of attaching antibody molecules to gold surfaces was to utilize the strong binding affinity between biotin and NeutrAvidin. Sulfo-NHS-SS-Biotin (Sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate) was first grafted to the gold surface of the cantilevers by the Au−S bond. NeutrAvidin could easily conjugate to the Sulfo-NHS-SS-Biotin on the gold surface due to the strong affinity between biotin and NeutrAvidin ($K_a = 10^{15}$ M^{-1}). The anti-PSA antibody was biotinylated for the subsequent coupling through NeutrAvidin. Greater detail regarding the protocols for surface functionalization and passivation can be found in the Supporting Information section.

After passivation and immobilization, the chip was mounted in the optical detection system as previously described.[12,13] A collimated laser beam reflected from each cantilever was collected by the CCD camera, so that each cantilever's motion was recorded during the entire reaction by monitoring of its respective reflected light spot. Base lines of the cantilevers' motion were recorded before any injection of antigen solution. Our model targets consisted of fPSA or BSA dissolved in physiologic PBS at specific test concentrations (see results). To inject the target solution to a given fluidic chamber, the PBS inside the chamber (reaction well) was first carefully aspirated using a micropipette. Next, the target solution was immediately pipetted into the aspirated well. The cantilever deflection was then monitored.

Figure 3 illustrates typical cantilever responses during the immobilization of crosslinkers. Injection itself did not cause deflection of the cantilevers, demonstrated by PBS injection in Figure 3A. Self-assembly of DTSSP on cantilevers induced a surface stress change of 20−40 mJ/m^2. In comparison, the binding of NeutrAvidin to the biotin on the cantilever surface changed the surface stress of the cantilevers by about 30 mJ/m^2. As for the third functionalization alternative, we had to keep the chip immersed in the NHS-thiol solution in a sealed chamber due to the high volatility of ethanol, and hence no real-time cantilever response to the NHS-thiol immobilization could be recorded.

Figure 4 shows the cantilever responses to antibody−antigen binding for each of these combinations. The results clearly demonstrated that cantilevers responded consistently to the antibody−antigen binding when DTSSP or the mixture of NHS-thiol and PEG-thiol was used in the crosslinking method and PEG-silane as the passivation media. Comparison of Figure 4a,c, where the only difference was BSA passivation of the silicon nitride surface as opposed to PEG silane passivation, suggests that BSA was inadequate to prevent nonspecific binding and that PEG silane was more effective. Owing to the inadequacy of passivation with BSA, we did not test BSA in combination with the other two functionalization chemistries. The poor performance of functionalization using the combination of biotin−NeutrAvidin (Figure 4b) may be due to the indirect, two-step coupling compared to the other cases, where only one crosslinker is used. Here, the two-step coupling likely results in a reduction in the density of available antigen-binding sites. Our results suggest that the efficacy of binding antibody to the surface decreases with increasing number of crosslinking steps. This reasoning can also explain why lower antigen density was observed when the NeutrAvidin−biotin method was used in comparison with DTSSP method.[18] Furthermore, the existence of NeutrAvidin increases the distance between the antibodies and the cantilever surface,

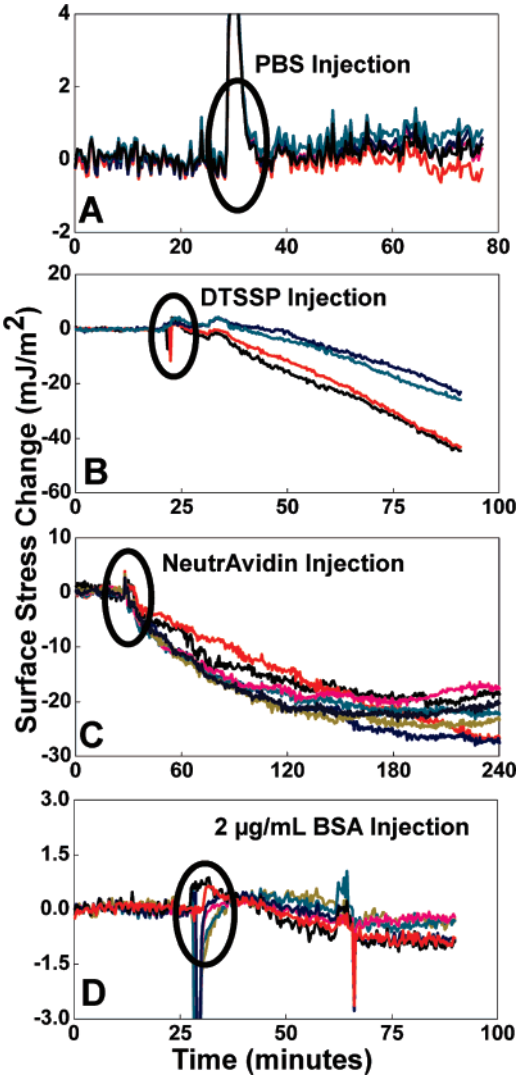

Figure 3. (A) Negligible deflection was caused by the PBS injection. The cantilevers were previously immersed in PBS, while PBS was introduced at time of 30 min. (B) Cantilever response of four cantilevers in two individual wells to DTSSP immobilization on gold surface. DTSSP was injected at minute 20. Negative surface stress change represented that a cantilever bent away from its gold surface. (C) Cantilever response to the NeutrAvidin binding to the immobilized biotin on the gold surface. Responses of seven cantilevers from three individual reaction wells were shown. The injection was done at minute 30; (D) BSA introduction only caused about surface stress change of 0.5 mJ/m^2. Anti-PSA was immobilized on the gold surface of the cantilevers by DTSSP. The other surfaces were passivated by PEG-silane.

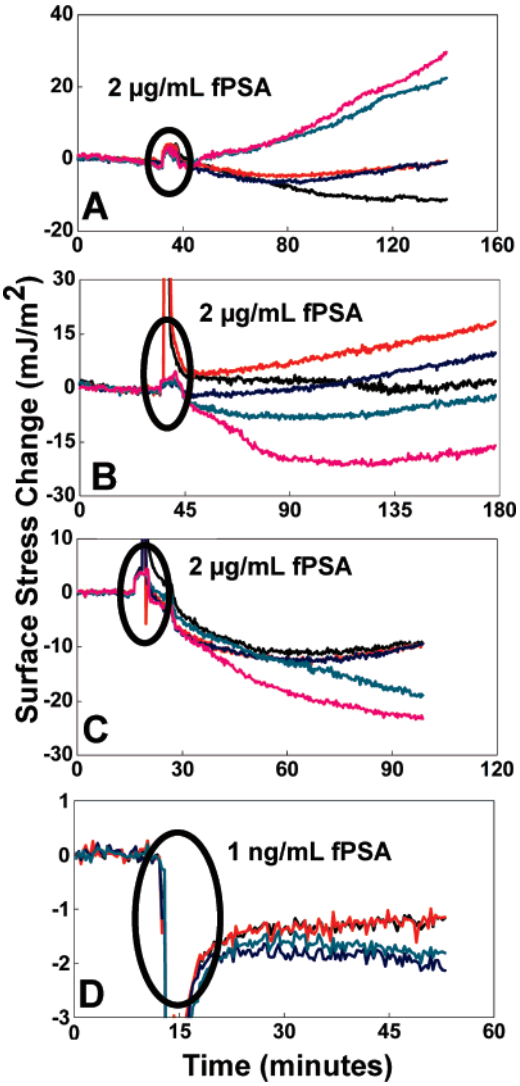

Figure 4. The monoclonal anti-PSA antibody was immobilized on the gold surface using various crosslinking methods and the silicon nitride surface was passivated using different blocking molecules. Cantilever response was monitored while fPSA solution was injected. (A) DTSSP linker (functionalization) and BSA (passivation); (B) Biotin−NeutrAvidin affinity (functionalization) and PEG-silane (passivation); (C) DTSSP linker (functionalization) and PEG-silane (passivation); (D) Mixture SAM (functionalization) and PEG-silane (passivation).

which may reduce the mechanical transduction efficiency of molecular interactions to cantilever surface stress change.

We measured the cantilever response to the binding of fPSA to the immobilized anti-PSA antibody for various PSA concentrations. As the antigen concentration decreased, the surface stress change resulting from the antibody−antigen binding also decreased (Figure 5). Arrays of 200 μm long cantilevers were used for the measurements when the fPSA concentrations were higher than 1 μg/mL, while arrays of 400 μm long cantilevers were used for lower fPSA concentrations. To detect lower PSA levels of 1 and 10 ng/mL, the mixture of NHS-thiol and PEG-thiol was used for immobilization of the antibody, while for the higher PSA concentrations DTSSP was used. The specific binding with 1 ng/mL of fPSA produced surface stress of about 2 mJ/m^2,

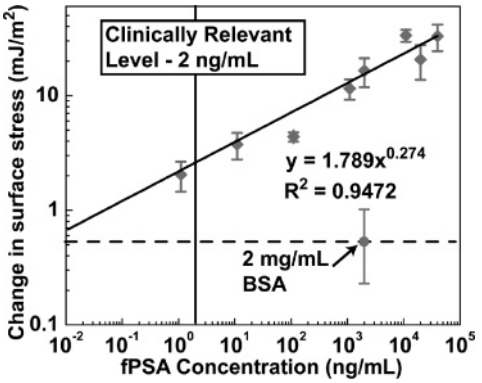

Figure 5. Response of 50 cantilevers (2−12 cantilevers per point) to antibody−antigen binding (PSA) on the gold surface reduced as the concentration of fPSA decreased. Error bar represents plus and minus one standard deviation around the mean. Nonspecific binding caused by the injection of 2 mg/mL BSA only resulted in surface stress change of 0.5 mJ/m^2.

while the nonspecific binding of 2 mg/mL BSA to the anti-PSA antibody caused almost negligible surface stress change, ~0.5 mJ/m^2. Given the correlation between the cantilever response and PSA concentration, the cantilever response resulting from BSA injection was approximately equal to the projected cantilever response to 0.01 ng/mL PSA. In this way, with a signal-to-noise ratio of about 2, one can expect the detection limit of PSA concentration is 0.2 ng/mL using the cantilever platform, an order of magnitude below the suggested 2 ng/mL clinical cutoff level.[19]

In conclusion, we have developed a label-free antibody−antigen binding assay in a multiplexed format using two-dimensional microcantilever arrays. Prostate specific antigen was chosen as the model analyte. We investigated immobilization of the captured antibody molecules onto the gold surface of cantilevers utilizing three different cross-linkers, including DTSSP, NHS-thiol, and biotin−NeutrAvidin. We examined the passivation of the silicon nitride surface by either BSA or PEG-silane. Consistent cantilever responses to antibody−antigen binding were achievable only when DTSSP or NHS-thiol was used as the crosslinker and PEG-silane was used as the passivation agent. Quantitative detection of PSA as low as 1 ng/mL was demonstrated using an array of 400 μm long cantilevers, which yielded 2 mJ/m^2 surface stress change due to the binding of the antibody and antigen. When 2 mg/mL BSA was present, the nonspecific binding resulted in surface stress change of only 0.5 mJ/m^2. Because the nanomechanical transduction from biomolecular reactions to the mechanical stress change is universal, we believe that such 2D microcantilever arrays may be utilized to develop real-time, label-free multiplexed assays with high sensitivity for detection of biomolecular "profiles" characterizing cancer as well other devastating diseases.

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Supporting Information Available: Materials and methods of immobilization of capture molecules and passivation.

This material is available free of charge via the Internet at http://pubs.acs.org.

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