



Label-Free Detection of Aflatoxin B1 Using a Nanomechanical Sensor

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Abstract: As a highly sensitive nanomechanical sensor, microcantilever sensor is widely used in biochemical detection. Aflatoxin B1 (AFB1), a hepatocarcinogen widely present in food and food materials, is highly dangerous to human health, and new sensitive methods to detect AFB1 are needed. Here, we developed a microcantilever-array-based immunosensor used in stress mode to detect AFB1 with the obvious advantages of a high sensitivity, rapidity, label-free, quantitative, and ability to be performed in real-time. The microcantilever was functionalized with a sulfhydrylated anti-AFB1 antibody, and an ELISA was used to validate the activity of the antibody on the microcantilever. Deflection of the microcantilever corresponding to different AFB1 concentrations was monitored in real-time. The detection limit of the microcantilever sensor in stress mode was lowered to 0.03 ng/mL for AFB1, which is a significant improvement in comparison with icELISA or a microcantilever sensor operated in dynamic mode. We also successfully detected AFB1 dissolved in a peanut solution. The microcantilever sensor in stress mode provides a new method for detecting extremely low concentrations of AFB1 and may have great potential for food quality control and public health protection.

Keywords: Microcantilever, Nanomechanical Sensor, Aflatoxin B1, Label-Free, Surface Stress

1. Introduction

Aflatoxin B1 (AFB1), produced primarily by *Aspergillus flavus* and *Aspergillus parasiticus*, is the most potent natural hepatocarcinogen and has been designated as a human liver carcinogen (group I) by the International Agency for Research on Cancer [1–3]. AFB1 has been reported in a variety of food and food materials and mainly enters the food chain through the dietary route in humans and animals [4, 5]. The intake of AFB1 over a long period of time, even at very low concentrations, may greatly damage human health [6]. The European Commission has set the maximum permissible level of AFB1 in food to 2 ng/g [7]. Therefore, the development of simple and sensitive techniques to detect low concentrations of AFB1 with quick response times is urgently required.

Many analytical methods are currently available to detect AFB1, including thin layer chromatography [8, 9] and

high-performance liquid chromatography [10–12]. Although these techniques are extremely sensitive, they typically require extensive sample pretreatment, tedious procedures and expensive equipment. The enzyme-linked immunosorbent assay (ELISA) is a highly sensitive and selective method for AFB1 detection [13, 14] and is widely used. However, small molecules need to be labeled for this technique, which is often difficult and may affect the sensitivity of the assay.

The microcantilever sensor, a highly sensitive, label-free, real-time, rapid-response, and efficient nanomechanical sensor, may be advantageous [15]. When the microcantilever is operated in stress mode, the stress induced by binding events on its surface causes the microcantilever to bend. The microcantilever, which is functionalized with receptor molecules, can recognize the corresponding analyte, including dangerous pesticides [16, 17], proteins [18, 19], heavy metal ions [20, 21], drugs [22–24], explosives [25, 26],

and bacteria [27, 28]. The microcantilever can also be operated in dynamic mode, during which the adsorption or release of a mass alters the resonant frequency of the microcantilever, and this mode is usually used to detect macromolecules [29, 30]. However, the microcantilever in stress mode can be used to detect not only macromolecules [18, 19] but also small molecules [22, 23].

Here, we developed a nanomechanical immunosensor to detect AFB1 using the microcantilever array in stress mode. An anti-AFB1 antibody was reacted with a sulfhydrylation reagent to produce a thiol (-SH) group, through which it was immobilized onto the gold surface of the microcantilever. The antibody-functionalized microcantilever was then used to detect different concentrations of AFB1. An ELISA was used to test the activity of the anti-AFB1 antibody that was immobilized on the microcantilever surface.

2. Materials and Methods

2.1. Materials and Apparatus

The anti-AFB1 monoclonal antibody, 2-iminothiolane hydrochloride, bovine serum albumin (BSA), goat anti-mouse IgG conjugated with horseradish peroxidase (IgG-HRP), aflatoxin G2 (AFG2) and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). AFB1 and an aflatoxin B1-ovalbumin conjugate (AFB1-OVA) were purchased from the Beijing Hapten and Protein Biomedical Institute (Beijing, China). The buffers and solutions used included phosphate-buffered saline (PBS; 0.1 M phosphate buffer containing 0.9% sodium chloride, pH 7.5), PBS with 0.1% (v/v) Tween 20 (PBST), and stop solution (2.0 M sulfuric acid).

The 96-well polystyrene microplates were purchased from Costar (Corning, NY, USA). The automated plate-washer (Wellwash 4 MK2) and the microplate reader (Multiskan MK3) were purchased from Thermo (Vantaa, Finland). Silicon microcantilever arrays (Micromotive GmbH, Mainz, Germany) were used. Each array contained eight identical microcantilevers, and the dimensions of each microcantilever were $500 \times 90 \times 1 \mu\text{m}^3$. One side of the microcantilever had a thin film of titanium (2 nm) covered with a 20 nm layer of gold. An experimental device received from Concentris GmbH (Switzerland) was used for microcantilever array immunosensor detection.

2.2. Microcantilever Array Functionalization

The sulfhydrylation protocol for the anti-AFB1 antibody was the same as previously described [20, 22]. Briefly, 48.58 μL of a 2.0 mg/mL 2-iminothiolane hydrochloride solution was added to 1.0 mL of a 10 mg/mL anti-AFB1 antibody solution. After reacting for 1 h at room temperature, the mixture was dialyzed against 20 mM PBS buffer (containing 150 mM NaCl and 1.0 mM EDTA, pH 7.2) for 48 h. The dialyzed sulfhydrylated anti-AFB1 antibody was stored at -40°C .

The procedure to immobilize the sulfhydrylated anti-AFB1

antibody onto the microcantilever array was performed in a microplate well, which was replaced after each step. A new microcantilever array was used in each experiment. Before functionalization, the microcantilever array was immersed in 'piranha dip' [$\text{V}(\text{H}_2\text{O}_2):\text{V}(\text{H}_2\text{SO}_4) = 1:3$] for 4 min, washed four times with deionized water and dried under a gentle stream of nitrogen gas. The array was then immersed in 200 μL of a 50 $\mu\text{g}/\text{mL}$ sulfhydrylated anti-AFB1 antibody solution and incubated for 2 h at 37°C for functionalization. The functionalized microcantilever array was washed four times with PBST and was then ready to use.

2.3. Detection of AFB1 Using Indirect Competitive Enzyme-Linked Immunosorbent Assay (icELISA)

IcELISA was used as the control method to detect AFB1. A microplate was coated with 100 μL of a 1 $\mu\text{g}/\text{mL}$ AFB1-OVA solution for 3 h. After the microplate was washed four times with PBST, it was blocked with 100 μL per well of a BSA (5% w/w) solution for 30 min and washed four times with PBST. Samples (100 μL) of different concentrations of the AFB1 solution were then added to the microplate wells, followed by 100 μL of a 150 ng/mL anti-AFB1 antibody solution. After being incubated for 30 min, the microplate was washed four times with PBST, and then 100 μL of an IgG-HRP solution was added to each well for 30 min. After the wells were washed four times with PBST, 100 μL of a TMB substrate solution was added to each well for 20 min. The reaction was stopped by adding 100 μL of stop solution per well. The absorbance B of the microplate was read at 450 nm with a microplate reader.

2.4. Characterization of the Activity of the Antibody Immobilized on the Microcantilever with ELISA

The activity of the anti-AFB1 antibody immobilized on the microcantilever array was characterized with an ELISA. The microcantilever array was placed in a microplate well, which was replaced after each step. As shown in Figure 3(A), the functionalized microcantilever array (a bare microcantilever array was used as the control) was placed in a microplate well, 200 μL of a BSA (5% w/w) solution was added, and the microplate was incubated at 37°C for 30 min. After the well was washed four times with PBST, 200 μL of an IgG-HRP solution was added, and the microplate was incubated at 37°C for 30 min. Any unbound enzyme conjugate on the microcantilever array was removed by washing the well four times with PBST. After the microcantilever array was placed in a new well, 100 μL of TMB was added to the well for 30 min, and the reaction was stopped by adding 100 μL of stop solution. The microcantilever array was removed, and the absorbance of the remaining solution was read at 450 nm with a microplate reader.

2.5. Microcantilever Array Immunosensor Detection

The functionalized microcantilever array was mounted in a fluid cell (5 μL), as shown in Figure 1. PBS solution was injected into the cell with a peristaltic pump. The flow was

maintained at a constant rate (0.54 mL/h) after PBS was injected. All the injected liquids was preheated to 300 ± 0.01 K. The temperature of the cell was maintained at 300 ± 0.01 K. And the room temperature was maintained at 300 ± 1 K. The deflection of the microcantilever was measured by monitoring the positions of laser beams reflected from the microcantilever to a position-sensitive detector. After deflection of the microcantilevers in array was equilibrated in running buffer, AFB1 dissolved in PBS was injected into the reaction cell, and deflection of the microcantilever array was monitored.

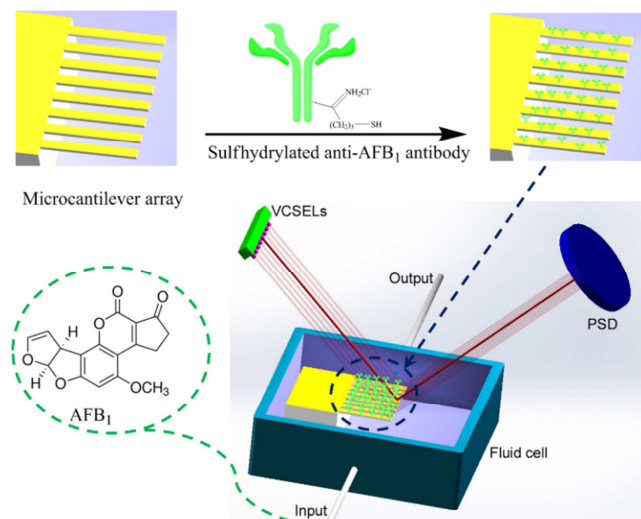


Figure 1. Schematic diagram of the microcantilever array immunosensor and the microcantilever array immobilized with antibody. VCSELs, vertical cavity surface emitting lasers; PSD, position sensitive detector.

2.6. Preparation of Peanut Samples

Peanuts (20 g, purchased in a local market) were immersed in 500 mL of a PBS solution for 24 h, and the peanuts were then removed from the solution. The remaining solution was filtered sequentially through filter papers with pore sizes of 10, 5, 1.2, and 0.22 μm . The filtered solution was called the “peanut solution”. Different amounts of standard AFB1 were added to the peanut solution to generate different concentrations of AFB1.

3. Results and Discussion

3.1. Detection of AFB1 with icELISA

AFB1 (MW = 312 Da) is the most potent natural hepatocarcinogen and is recognized as a potential threat to human health, even at low concentrations. The microcantilever array immunosensor was designed to detect low concentrations of AFB1. Before detection, an icELISA was used as a control method to detect AFB1 and measure the binding activity of the anti-AFB1 antibody to AFB1. The measured inhibition curve for AFB1 is shown in Figure 2. B_0 and B are the absorbances in the absence and presence of competitor, respectively. The B/B_0 ratio decreased as the AFB1 concentration increased, indicating the inhibitory

effect of AFB1 on the binding of AFB1–OVA to the anti-AFB1 antibody. This confirmed the ability of the anti-AFB1 antibody to bind AFB1. The sensitivity of AFB1 detection by icELISA, which was calculated as the concentration that led to 20% inhibition, was 0.81 ng/mL.

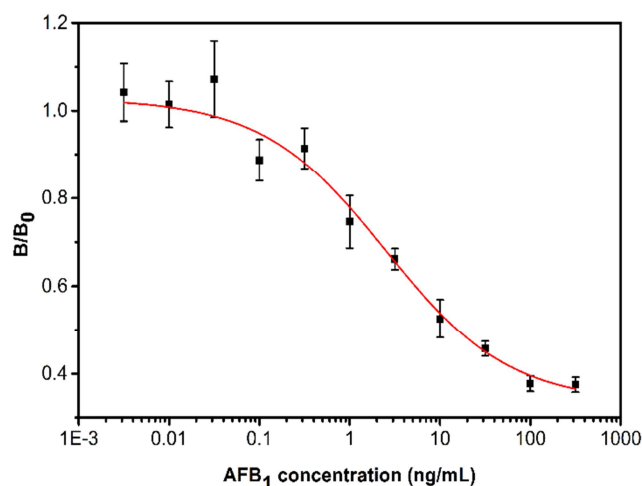


Figure 2. Inhibition curve for AFB1 in an icELISA. The concentration that led to 20% inhibition by AFB1 was 0.81 ng/mL.

3.2. Activity of the Antibody on the Microcantilever

To detect AFB1, the microcantilever must first be modified with a receptor molecule, such as an anti-AFB1 antibody. To confirm that the antibody was successfully immobilized on the microcantilever surface and remained active, an ELISA was used to test its ability to bind to goat anti-mouse IgG (details in Materials and methods). As shown in Figure 3(B), the absorbances B of the functionalized and naked microcantilevers were 0.235 and 0.052, respectively, which indirectly verified successful functionalization of the microcantilever array with sulhydrylated anti-AFB1 antibody and confirmed that the antibody remained active.

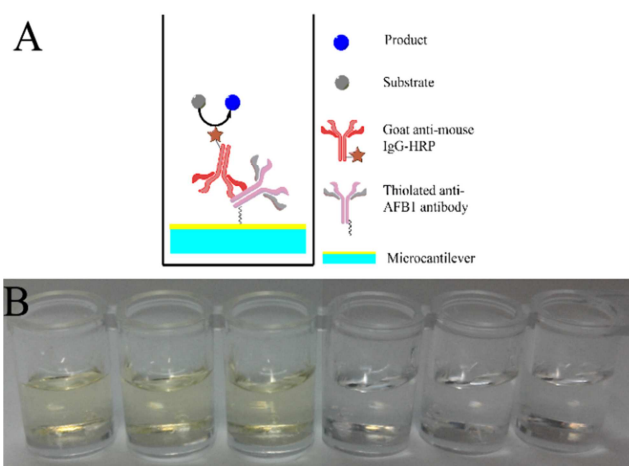


Figure 3. (A) Schematic diagram of the immunological reactions on the microcantilever arrays using ELISA. (B) Characterization of the anti-AFB1 antibody on the microcantilevers. The absorbance B of the anti-AFB1-antibody-functionalized microcantilever (1, $B = 0.235 \pm 0.003$) was compared with that of the naked microcantilever arrays (2, $B = 0.052 \pm 0.002$).

3.3. Detection of AFB1 by the Microcantilever Array Immunosensor

Immobilization of the antibody on the microcantilever is important to ensure the sensitivity and stability of the microcantilever immunosensor. Here, the antibody was immobilized onto the gold surface of the microcantilever with the following method. The antibody was sulfhydrylated with the sulfhydrylation reagent 2-iminothiolane hydrochloride, which reacted with the $-NH_2$ group on the antibody to produce a thiol ($-SH$) group through which the antibody was conjugated to the gold surface [20, 22]. This method required only a single step for antibody immobilization and was highly effective.

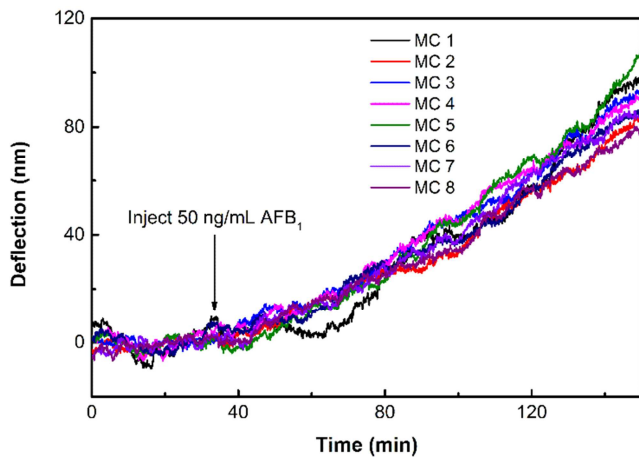


Figure 4. Real-time deflection of each microcantilever in an array in response to 50 ng/mL AFB1. MC, microcantilever.

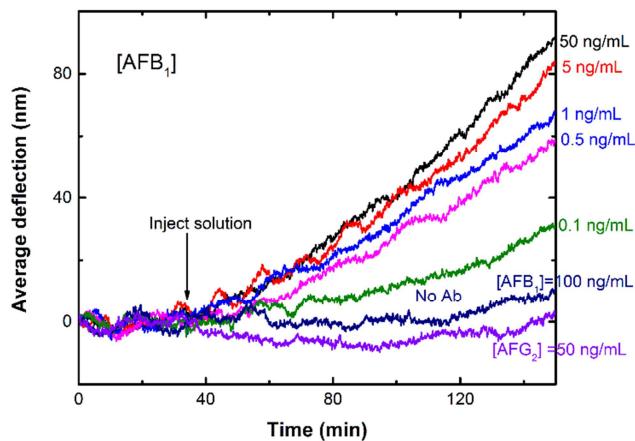


Figure 5. Average microcantilever deflections as a function of time at various concentrations of AFB1. The microcantilever arrays were functionalized with a sulfhydrylated anti-AFB1 antibody. The average deflection represents the mean deflection of the identically functionalized microcantilevers within an array.

For a microcantilever array sensor, the deflection of identically treated microcantilevers must be the same. To examine the response consistency of the different microcantilevers in an array, we measured the deflection curves of all of the microcantilevers within an array in response to 50 ng/mL AFB1. All of the microcantilevers in

the array were functionalized with the AFB1 antibody. After baseline deflection of the microcantilever array was stabilized in PBS for 33 min, 50 ng/mL AFB1 solution dissolved in PBS was injected, as shown in Figure 4. Deflections of the different microcantilevers in the array were very similar (91.5 ± 9.1 nm), indicating that the microcantilever array immunosensor had good consistency for detecting AFB1.

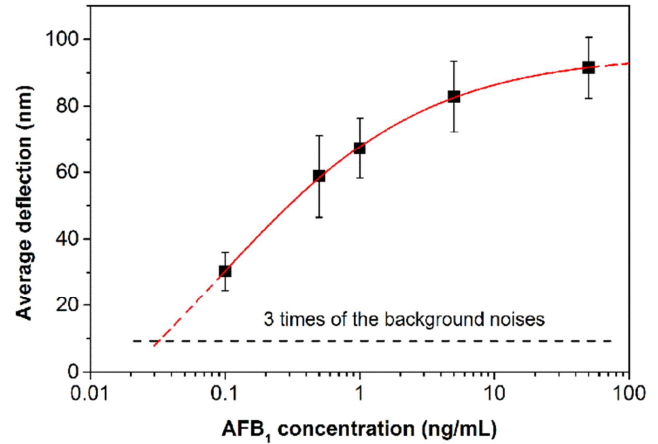


Figure 6. Maximum deflection of the microcantilever array at various concentrations of AFB1.

To investigate the relationship between microcantilever deflection and AFB1 concentration, we measured microcantilever deflections induced by different concentrations of AFB1. After the baseline of microcantilever deflection was stabilized in PBS solution, different concentrations of AFB1 dissolved in PBS were circulated through the fluid cell, respectively. The deflection curves of the microcantilevers in response to various AFB1 concentrations are shown in Figure 5. The average deflection represents the mean bending value of identically functionalized microcantilevers within an array. We defined microcantilever bending toward the gold layer as positive (up). The binding between the anti-AFB1 antibody and AFB1 caused the microcantilever to bend toward the gold surface so that tensile stress occurred on the gold surface. The average bending signals at 150 min for AFB1 solutions of 50, 5, 1, 0.5, and 0.1 ng/mL were 92, 83, 67, 59, and 30 nm, respectively. When the reference microcantilever without antibody was exposed to 100 ng/mL AFB1, the microcantilever did not show a significant response. Moreover, a significant response was also not observed when 50 ng/mL AFG2, which is a toxin in food and food products but does not react with anti-AFB1 antibody, was added to the cell. These results indicated that the bending of the microcantilever array was caused by the specific binding interaction between the anti-AFB1 antibody and AFB1.

The maximum microcantilever deflection caused by the specific binding of the anti-AFB1 antibody to AFB1 in the test period is shown in Figure 6. Each data point and error bar represents the mean deflection value and standard deviation, respectively, for identically functionalized

microcantilevers within an array. The responses of the microcantilever array showed a positive correlation between the deflection and the concentration of AFB1, indicating that the microcantilever can quantitatively detect the analyte. The background noise of the measurement was approximately 3 nm, so the limit of detection (LOD) of the microcantilever sensor, which was calculated as three times higher than the background noise, was approximately as low as 0.03 ng/mL AFB1. Our results demonstrated that the microcantilever operated in stress mode was sensitive and suitable to detect AFB1.

The surface stress generated by the binding interaction between a small molecule and its antibody was a tensile stress in the present study, which differs from the compressive stress induced by the binding interaction between a macromolecule and its antibody [17, 31, 32]. Our findings are consistent with the results of another study [17]. The stress on the microcantilever surface may have originated from the conformational changes induced in the antibody when it bound to the antigen, manifesting as the motion of the Fab arm, which exposed the C1q-binding site on the antibody, as proposed by Wu *et al.* [17]. This generated a stress difference between the AFB1-antibody-modified surface on the upper side of the microcantilever and the silicon surface on the lower side.

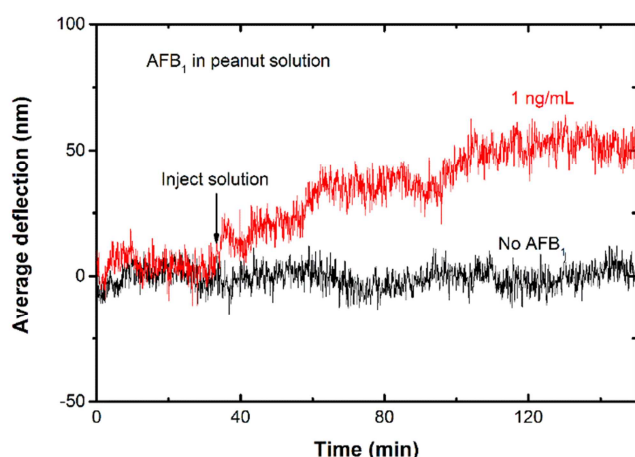


Figure 7. Real-time average deflection of the microcantilever array for AFB1 dissolved in a peanut solution.

A microcantilever sensor operated in dynamic mode was also used to detect total aflatoxins with an LOD of 3 ng/mL, as reported by Ricciardi *et al.* [33]. The LOD of icELISA for AFB1 was 0.81 ng/mL (Section 3.1). Therefore, compared with other methods, the microcantilever immunosensor in stress mode that we developed here showed a significant improvement in sensitivity. The large sensitivity difference between the microcantilever in stress and dynamic modes is interesting to investigate. The frequency shifts of microcantilevers in dynamic mode are mainly caused by added mass, and this mode is usually used to detect macromolecules [29, 30, 34]. However, the weight of a molecule, such as AFB1 (MW = 312 Da) is so small that the added mass to the microcantilever was low, and thus the

frequency shift of the microcantilever induced by adsorption of a small amount of AFB1 may not have been large enough. Therefore, the microcantilever in dynamic mode showed a lower sensitivity for AFB1. The sensitivity of the microcantilever for AFB1 in the present study demonstrated a 100-fold improvement in comparison with the microcantilever in dynamic mode. The deflection of the microcantilever in stress mode was not determined by the adsorbed mass but may be attributed to the conformational changes in the anti-AFB1 antibody that were induced when it bound to AFB1, which may have caused a large energy release from the anti-AFB1 antibody, similarly to that released from a compressed spring. Therefore, trace amounts of AFB1 can cause sufficient deflection of the microcantilever, and thus the microcantilever in stress mode showed higher sensitivity.

3.4. Detection of AFB1 in Peanuts

AFB1 that is present in peanuts has become a threat to human health. Therefore, we used the microcantilever array immunosensor to detect AFB1 dissolved in a peanut solution. The average deflection profile of the microcantilever for AFB1 in a peanut solution is shown in Figure 7. The average deflection of the microcantilevers at 150 min was approximately 51 nm for 1 ng/mL AFB1 in the peanut solution, which was close to the deflection (approximately 67 nm) for 1 ng/mL AFB1 in PBS. No significant response was observed when the peanut solution without AFB1, which was used as a reference, was injected into the cell. The fluctuations in the mean deflection induced by the injection of AFB1 dissolved in the peanut solution (Figure 7) were greater than the fluctuations in the deflection induced by the injection of AFB1 dissolved in PBS (Figure 5), which may indicate that the simply treated peanut solution contained more complex substances than the PBS solution. These data show that the microcantilever immunosensor can detect AFB1 in peanut solutions that contain complex components, indicating that the microcantilever can be used for on-site AFB1 detection.

4. Conclusion

In conclusion, we showed that the microcantilever array operated in stress mode can sensitively detect AFB1 without the need for a label in both PBS and peanut solutions. A sulfhydrylated anti-AFB1 antibody was immobilized onto the gold surface of the microcantilever and an ELISA was used to validate the activity of the antibody on the microcantilever. The deflection increased with increasing concentrations of AFB1, indicating that the microcantilever sensor can quantitatively detect AFB1. The LOD of the microcantilever operated in stress mode for AFB1 was as low as 0.03 ng/mL, which is a significant improvement in comparison with icELISA (0.81 ng/mL) or a microcantilever operated in dynamic mode (3 ng/mL). The microcantilever sensor was successfully used to detect AFB1 in a peanut solution, showing its capacity to detect the analyte on site in a

complex environment. The results indicate that the microcantilever in stress mode is well suited to detect small molecules with significantly low LOD. Passivation of Si surface, on-chip reference and antibody immobilization, however, may affect the detection sensitivity and reliability. Thus, further research should focus on these.

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