Highly Sensitive Nanomechanical Immunosensor Using Half Antibody Fragments

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ABSTRACT: The improvement of sensitivity is of great significance to the application of biochemical sensor. In this study, we propose a micocantilever-based immunosensor in surface stress mode using half antibody fragments as receptor molecules. The thiol-containing half antibody fragment was obtained with a low loss of antibody biological activity and then was covalently and orientedly immobilized on the gold surface of microcantilevers via two native thiol groups. Such a one-step reaction and immobilization of receptor molecule simplify the preparation process of micocantilever immunosensor. Using shortened and highly oriented half antibody fragments as receptor molecules, the generation of surface stress and the transmission of stress from the interaction region of molecules to the surface of the microcantilever have been elevated significantly. The limit of detection (LOD) of the presented sensor has been significantly lowered to 1 pg/mL, or 1.1 pM in equivalence, which is a 500-fold improvement when compared with intact full antibody coated conventional micocantilever sensors. The results indicate that the half antibody fragment is well suited for the functionalization of the microcantilever surface and is generally applicable to all microcantilever immunosensor development, and this principle will help to design a functional film of devices with significantly lower LOD.

Microcantilever, as a biochemical sensor, has attracted more and more interest for its highly sensitive, label-free, and real-time detection.1,2 In the past decade, the applications of the microcantilever sensors have been extended to the measurements of various molecular interactions, making it an adaptable and versatile sensor.1,2 The fundamental principle of the microcantilever operated in static or surface stress mode is that the interaction between target and the receptor molecules that are immobilized on only one side of the sensor generates a change of surface stress and, then, the change bends the microcantilever. Owing to the attractive features, such as label-free detection and high sensitivity, microcantilever sensors have been successfully applied to detect various molecular interactions such as proteins, DNA, lipids, pesticides, and heavy metal ions.2−13 A molecule, as an antigen, was detected using a microcantilever immunosensor, where its specific antibody was immobilized on one side of sensor as a receptor molecule.14−18

It is well-known that the immobilization of antibody (receptor molecule) on the sensor surface is a critical step for the preparation of immunosensor. It is crucial to orient the antigen-binding sites of the antibodies toward the analytes. For sensors with a gold-coated surface, self-assembled monolayers (SAM) of thiolated molecules containing a reactive functional group are often used. However, the orientation of antibodies that are immobilized using these approaches is random, which leads to the loss of binding capability due to steric-hindrance effect.19−21 In order to explore the full potential of the immunosensors, it is necessary to develop a proper antibody immobilization method to ensure a uniformed antibody orientation with the antigen-binding sites toward the analytes. Correctly and uniformly oriented antibodies minimize the steric hindrances22 and allow antigens to bind more easily. The oriented antibodies provide 2−8 times higher binding capacity to antigens and, thus, make the immunosensor more sensitive.23

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As previously reported, the sensitivity of microcantilever-based immunosensors can be improved by both oriented immobilization of antibodies and reduced distance between the antigen-binding Fab regions and the microcantilever surface. As in other immunosensors, the oriented immobilization can enhance the binding capacity of antibody to antigen in microcantilever immunosensors, and more importantly, the stress generated from the binding where the antibody is orientedly immobilized is in the same direction and thus can lead to a greater resultant force, which would greatly improve the sensitivity of the microcantilever sensor whose principle is transduction of the force into a bend of the microcantilever.

Shortening the distance between the antibody and the microcantilever surface results in significantly elevating the transmission of stress from the reaction region in antigen−antibody to the surface of the microcantilever, thus increasing the sensitivity of the microcantilever sensor by a factor of 100. An antibody is a Y-shaped protein molecule that is composed of two identical halves which are combined together by two disulfide bonds (Figure 1). The antibody can be split into two identical half fragments by reducing these disulfide bonds; meanwhile, reduction of the two disulfide bonds introduces two thiol groups to each half fragment. Thus, oriented immobilization of half fragment on gold surface can be achieved because the thiol groups are in a fixed position at the far end that is away from the Fab region. Consequently, the detection sensitivities of quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) can be greatly increased using this fragment as receptor molecule due to its sufficient antigen binding capacity. However, SPR and QCM cannot directly detect small molecule due to its small volume and small molecular weight. On the basis of the principle of surface stress, microcantilever sensors not only can detect macro-molecule but also can directly detect small molecule with high sensitivity. However, there have not been any related studies about the application of half antibody fragment on microcantilever immunosensor. For microcantilever immunosensor which is based on stress effect, we can speculate that highly oriented immobilization of half fragment provides not only high antigen binding capacity but also a greater resultant force due to the same direction of the stress generated from the binding. More importantly, using half fragment leads to the greatly shortened distance between the antigen-binding Fab regions and microcantilever surface (Figure 1). These factors raise the possibility that the utilization of highly oriented half antibody on microcantilever surface can greatly improve the generation and the transmission of surface stress to the microcantilever structure; subsequently, the sensor’s sensitivity can be enhanced significantly.

Here, we report the first attempt of using half antibody fragments as the active sensing molecule aiming to develop the next generation microcantilever-based immunosensor with ultrahigh sensitivity. An antibody with specificity to Ginsenoside Re (GRe) was used for obtaining the half antibody fragment. An enzyme-linked immunosorbent assay (ELISA) was used to evaluate the activity of half antibody fragment. The sensitivity of the presented method using half antibody fragments as receptor molecules was compared with previous studies using another oriented immobilization method to coat the same full antibodies on the microcantilever surface via goat antimouse IgG. In order to demonstrate the usefulness and adaptability of the presented technique, half antiabscisic acid

![Figure 1. Schematic of the microcantilever-based sensing system and oriented immobilization of half antibody fragments on the gold (Au) side of the microcantilever via their own thiol groups (−SH): 1, preparation of half fragment antibody by reducing disulphide bonds using 2-mercaptoethylamine (2-MEA); 2, oriented immobilization of half antibody fragments via their own two thiol groups conjugated to the gold with a single-step.](image-url)
(half anti-ABA) antibody fragments were used to biofunc-
tionalize the microcantilever.

**EXPERIMENTAL SECTION**

**Reagents and Solutions.** 2-Mercaptoethylamine (2-
MEA), Ginsenoside Re (GRe), and abscissic acid (ABA) were
bought from Sigma (St. Louis, MO, USA). The anti-GRe and
anti-ABA monoclonal antibody (anti-GRe mAb and anti-ABA
mAb) were obtained from previous research.29 The rest of the
chemical reagents were bought from Beijing Chemical Reagents
Co. (Beijing, China). 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), PBST containing 0.5% (w/v) gelatin
(PBSTG), coating buffer (0.05 M carbonate buffer, pH 9.6),
and citrate-phosphate buffer (0.01 M citric acid and 0.03 M
monosodium phosphate, pH 5.5).

**Apparatus.** Silicon nitride microcantilevers with a V-shape
(0.6 μm × 20 μm × 200 μm Veeco Instruments, Plainview,
NY) were used. One side of the microcantilever has a thin film
of chromium (15 nm) covered with a 60 nm layer of gold
deposited by e-beam evaporation. The experimental setup used
for microcantilever detection was home-built allowing one to
read out single cantilevers as described in our previous studies.18,24,47,29 The 96-well polystyrene microtiter plate was
bought from Costar (Corning, NY). A microplate reader (Multiskan MK3) and an automated plate-washer (Wellwash 4
MK) were bought from Thermo (Vantaa, Finland).

**Preparation of Half Antibody Fragment.** The half
antibody fragment was prepared by reducing the intact
antibody with 2-MEA. The reduction protocol and the optimal
concentration of 2-MEA were the same as those described by
Kausaite-Minkstimiene et al.19 To remove unreacted 2-MEA,
the reaction mixture was dialyzed against 20 mM PBS bu-
ser (0.05 M carbonate bu-
ser, pH 9.6) for 48 h.
Half antibody fragment solution was diluted to 1.0 mg/
ch ml with PBS and stored at −40 °C.

**Half Antibody Fragment Immobilization.** The immobi-
lization procedure was performed in a microplate well that was
replaced by a new one after each step. Each microcantilever was
pretreated with a “piranha dip” (H2O2/H2SO4 = 1:3, 100 μL)
for 15 min and washed with deionized water three times. (CAUTION: "Piranha" solution reacts violently with organic
materials; it must be handled with extreme care.) The
microcantilever was dried under a gentle stream of nitrogen
gas and then immersed into 200 μL of 4.0 μg/mL half antibody
fragment solution diluted by PBSTG and incubated for 2 h at
37 °C. After washing with PBST three times, the microcan-
tilever was blocked with 200 μL of 3% nonfat dry milk coating
buffer for 30 min at 37 °C. The microcantilevers were washed
with PBST three times and dried; the functionalized micro-
cantilevers were ready to use. Different new microcantilevers
were used in each binding experiment.

**Characterization of Half Antibody Fragment Activity.**
The binding activities of the intact antibody and half antibody
fragment were tested by noncompetitive ELISA as described
below. Microplate was coated with 200 μL of 0.5 μg/mL
coating antigen in coating buffer for 3 h at 37 °C. After
washing with PBST four times, the plate was blocked with 200 μL per
well of 3% nonfat milk in PBS for 30 min at 37 °C and then
washed with PBST four times. To the microplate was added
100 μL of the same concentration (0.2 μg/mL) of the intact
antibody and half antibody fragment dissolved in PBS. After
incubation at 37 °C for 30 min, the plate was washed with
PBST four times, and then, an aliquot of 200 μL per well of
goat antimouse IgG-HRP (Fab specific) diluted in PBS was
added. After incubation at 37 °C for 30 min, the plate was
washed again with PBST four times, and then, 200 μL per well
of substrate solution was pipetted. The reaction was stopped
after 15 min at room temperature by adding 50 μL of the stop
solution. The absorbance was read at 450 nm on a microplate
reader.

**Microcantilever Immunosensor Detection.** The micro-
cantilever was mounted in a fluid cell (0.6 mL) (Figure 1). PBS
was injected into the cell by a peristaltic pump. The flow
maintained a constant rate (4 mL/h) after the cell was
filled with PBS. The temperature of the room and the cell were
maintained at 301 ± 0.1 K and 310.00 ± 0.01 K, respectively.
The microcantilever deflection was measured by monitoring
the position of a laser beam reflected from the tip of the
microcantilever onto a position sensitive detector (PSD). After
microcantilever deflection was stabilized, the analytes dissolved
in PBS were added into the fluid cell and microcantilever
deflection was monitored in situ.

**RESULTS AND DISCUSSION**

**Binding Activity of Half Antibody Fragment.** The 2-
mercaptoethylamine (2-MEA) was used to split the antibody
to two half fragments by reducing disulfide bridges between
the two heavy chains without affecting the antigen binding sites
(Figure 1). The antibody we chose was the mouse monoclonal
antibody (mAb) of Ginsenoside Re (GRe, MW = 947 Da). The
dissociation constant (Kd) of anti-GRe mAb is 6.7 × 10−12 M.
GRe is the main component of American ginseng; it not only has
anti-ischemic, antioxidant, and antidiabetic effects but also
enhances proliferation of CD4+ T cell.28 The 2-MEA solution
used was an optimal concentration.19 The unreacted 2-MEA is
removed from the mixture by dialyzing. To confirm binding
activity of half antibody fragment, both intact antibody and half
antibody fragment were tested for their bindings to
immobilized coating antigens using noncompetitive ELISA.
The results revealed that the reduction of anti-GRe antibody
caused about 18.7% loss of activity for binding antigen. The
reduction decreased optical density (OD) from 1.766 ± 0.052
to 1.435 ± 0.071; the data was obtained from 16 time
detections in the ELISA plate (Figure 2), and the OD of ELISA
plate without coating antigen was only 0.069 ± 0.007. These

![Image](326x141 to 563x246)

**Figure 2.** Characterization of binding activities of the intact anti-GRe antibodies and half anti-GRe fragments by ELISA. The activities of the half anti-GRe fragments (1. OD = 1.435 ± 0.052) are compared with those of intact anti-GRe antibodies (2. OD = 1.7662 ± 0.071) and microplates without coating antigen (3. OD = 0.069 ± 0.007). The colors confirm the low loss of the binding activity of half antibody fragment and a specific binding of half anti-GRe fragment to GRe.
findings confirm the low loss of the binding activity of antibody because of reductions by 2-MEA and a specific binding of half anti-GRe fragment to GRe.

**Microcantilever Imunosensor Detection.** After functionalized with half antibody fragments, the microcantilever was mounted in a fluid cell, and an optical lever was used to measure the deflection of microcantilever in real time (Figure 1). After the baseline of deflection was stable in a steady continuous flow using phosphate buffered saline (PBS) solution, antigen (GRe) solution with various concentrations was circulated into the cell sequentially. The deflection was monitored in situ for approximately 80 min. The deflection curves for different concentrations of antigens were illustrated (Figure 3a). Antigen binding caused the microcantilevers to bend, and the cantilever bent toward the Au side. An equilibrium-bending signal of ≈87, 75, 55, and 32 nm was obtained for 10, 1.0, 0.1, and 0.02 ng/mL antigen solutions, respectively. As a reference, 1000 ng/mL chlorsulfuron (a nonbinder molecule, C_{12}H_{12}ClN_{5}O_{4}S, MW = 357.8 Da) and 10 μg/mL BSA dissolved in PBS were injected in the fluid cell and the microcantilever had no significant bending, indicating that the bending is caused by specific binding of half frag-anti-GRe and GRe. The bending occurred because the binding of GRe to half frag-anti-GRe results in a difference in force between the antibody fragments coated surface on the top side and the silicon nitride surface on the backside of the microcantilever.

Figure 4 illustrates the equilibrium deflections of microcantilever for various GRe concentrations. Every data point was an average of 4 experiments (the data of the other 3 experiments are shown in Figure S1 in Supporting Information) using different microcantilevers, and the error bar reflects the range of deflections. The responses of the microcantilever suggested a positive correlation between the equilibrium deflection and the concentration of GRe. The corresponding microcantilever deflection is proportional to the logarithm of the concentration of antigen. The background noise of the measurement was approximately 3 nm, and the limit of detection (LOD) was calculated to be 3 times higher than the background noise; thus, the LOD of the presented technique was in the 1 pg/mL range (1.1 pM). Our results demonstrated that the microcantilevers based on stress effect using half antibody fragments as receptor molecules are sensitive enough to detect GRe and quantify the GRe.

In our previous studies, anti-GRe antibody, the same sample used for obtaining half antibody fragment in the present study, was immobilized on the gold surface of the microcantilever via another two methods: (1) random immobilization by sulfhydrylated antibody using the sulfhydrylation reagent which reacts with the −NH_{2} group of the antibody to give a thiol (−SH) group for conjugation onto the gold surface; (2) oriented immobilization using sulfhydrylated goat antimouse IgG which can specifically bind the Fc part of the antibody to achieve oriented immobilization of antibody.29
worth noting is that the film of goat antimouse IgG increases the distance between the antigen binding and the microcantilever surface. This point decreases the stress transmission from antigen–antibody reactions to microcantilever and reduces the microcantilever sensitivity. In the current study, using the half antibody fragment as the receptor molecule, the sensitivity had a 500-fold increase. The improvement of the sensitivity could be mainly attributable to higher density and higher efficiency of stress transmission from antigen–antibody reactions to microcantilever, which resulted from the single-step immobilization of half antibody fragments via two native thiol-groups directly conjugated to the gold and the shortened distance between the antigen-binding Fab regions and the microcantilever surface. In particular, there is only Fab part between the microcantilever surface and the antigen-binding site without linker molecule and Fc part (Figure 1), which greatly shortens the distance between the microcantilever surface and the antigen-binding site, resulting in a more efficient stress transmission. Also, immobilization via two thiol-groups enhances the stability of receptor molecule but, more importantly, increases the stiffness of the connection between the microcantilever surface and the receptor molecule, which in turn further improves stress transmission.

Intermolecular interactions governed by the Lennard–Jones potential, van der Waals, electrostatic forces, steric, as well as hydration, and surface hydrophobicity are considered to be the origins of the stresses. Antibody conformational change due to antigen binding was proposed as the origins for small-molecule antigen detection. The changes of conformation due to adsorbed molecules are also considered to be the origins in many other intermolecular interactions. Whatever the origin is, after generating in the antigen-binding Fab regions, the stress transmits through the receptor molecule and the molecule that links microcantilever surface and the receptor molecule and finally reaches and bends the microcantilever. Oriented immobilization and shorter distance between the antigen-binding Fab regions and the microcantilever surface with half antibody fragment as the receptor layer can enhance the generation of surface stress and the stress transmission for every kind of stress, regardless of its origin.

As previously reported, the sensitivity of a microcantilever immunosensor in surface stress mode using single-chain Fv (scFv) antibody fragment as receptor molecule was increased to \( \approx 1 \text{ nM} \). However, scFv fragments are obtained through genetic engineering rather than a conventional method and may not be easy to acquire since the production process is complex. Attaching a thiol group to the C-terminal end of scFv may not be easy to acquire since the production process is complex. In the present study, by immobilizing half antibody fragment via two native thiol-groups, which provides better stability of the receptor molecule and a higher stiffness of the connection between receptor molecule and microcantilever, we increased the sensitivity of microcantilevers at limits of detection (LOD) of 1.1 pM (1 pg/mL). Another important advantage is that thiol-containing half antibody fragment was obtained via a simple chemical reaction with only one step.

Usefulness and Adaptability of the Presented Technique. To demonstrate the usefulness and adaptability of the presented technique, a microcantilever immunosensor for phytohormone abscisic acid (ABA) was developed, using the procedure described above for the GRe microcantilever (Figure 1). ABA is an important phytohormone that has critical functions for growth and development and for stress resistance, and it regulates the expression of many genes in plants. The sensitivity of the method using half antibody fragment was compared with the microcantilever with antibody immobilized via thiolated anti-ABA antibody using the sulphydrylation reagent 2-iminothiolane hydrochloride which reacts with the \(-\text{NH}_2\) group of the antibody to give a thiol (\(-\text{SH}\)) group for conjugation onto the gold surface. Figure 5a shows the real-time deflections of the microcantilever coated with half anti-ABA fragment with the injection of different concentrations of ABA. As a reference, 1000 ng/mL chlorsulfuron (a nonbinder molecule, \( \text{C}_{12}\text{H}_{15}\text{ClN}_{2}\text{O}_8\text{S}, \text{MW} = 357.8 \text{ Da} \)) and 10 \( \mu \text{g/mL} \) BSA dissolved in PBS were injected in the fluid cell, and the microcantilever had no significant bending, indicating that the bending is caused by specific binding of half frag-anti-ABA and ABA. Figure 5b shows the real-time deflections of the microcantilever coated with thiolated anti-ABA antibody with the injection of different concentrations of ABA. In the same ABA concentration of 10 ng/mL, the deflections of the microcantilever with antibody immobilized via half anti-ABA fragment and thiolated anti-ABA antibody were \( \approx 125 \) and \( \approx 65 \) nm, respectively. The results indicated that the microcantilevers
coated with half anti-ABA fragment had a higher sensitivity than those with thiolated anti-ABA antibody.

**CONCLUSIONS**

Immobilization of receptor molecules on sensor surfaces is a difficult point, but a key point, especially for further application of the technique. In the current study, we investigated the application of half antibody fragment as receptor molecule for microcantilever sensor in surface stress mode. Half antibody fragment was chosen for its shorter distance between the antigen-binding Fab regions and the microcantilever surface significantly increases the stress transmission from the interaction region of molecules to the surface of the microcantilever. We demonstrated that using half antibody fragment as receptor molecule improved the sensitivity of microcantilever significantly. The current LOD of the method for GRe detection is as low as 1 pg/mL, which improved 500-fold compared with a previous study using intact antibody molecule. One-step reaction and immobilization of receptor molecule are two advantages of the current method. The results indicate that the half antibody fragment is well suited for the functionalization of microcantilever surface and is generally applicable to all antibodies in microcantilever sensor development. Further research should focus on improvement and preserving biological activity of antibody fragment on the gold surface of the microcantilever and reuse of microcantilever sensors.

**ASSOCIATED CONTENT**

Additional Information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org/.

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**Author Contributions**
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**REFERENCES**