Development of Protein A Functionalized Microcantilever Immunosensors for the Analyses of Small Molecules at Parts per Trillion Levels

Weiming Tan,† Yuan Huang,† Tiegui Nan,† Changguo Xue,‡ Zhaohu Li,† Qingchuan Zhang,*,† and Baomin Wang*,†

College of Agronomy and Biotechnology, China Agricultural University, Beijing 100193, People’s Republic of China, and Key Laboratory of Mechanical Behavior and Design of Material of Chinese Academy of Sciences, University of Science and Technology of China, Hefei 230027, People’s Republic of China

Development of microcantilever biosensors for small molecules was exemplified with the β-adrenergic agonist clenbuterol and the antibiotic chloramphenicol. In this paper, antibody sulfhydrylation and protein A were used to modify the microcantilever Au surface, and the antibody activities on the microcantilever were evaluated with direct competitive enzyme-linked immunosorbent assay (dcELISA). The activity of the antibodies immobilized on the microcantilever via protein A was 1.7-fold of that via the sulfhydrylation reagent 2-iminothiolane hydrochloride. A microcantilever immunosensor method with protein A as the functionalization reagent was established to detect the residues of clenbuterol and chloramphenicol at limits of detection (LOD) of approximately 0.1 and 0.2 ng/mL, respectively. Such LODs were better than that of the corresponding dcELISAs. The concentration of clenbuterol in a fortified feed sample detected with the corresponding dcELISAs. The concentration of clenbuterol and the antibiotic chloramphenicol. In this paper, protein A showed to be simple and reproducible and purification agreed well with that detected with the dcELISA. Protein A showed to be simple and reproducible for functionalization of the antibodies on the Au surface and, thus, has common application values in microcantilever immunosensor development. The results suggest that microcantilever immunosensors be suitable for detection of small molecules, and the assay sensitivity is mainly related to the quality and activities of the antibodies.

The microcantilever biosensor, benefiting from the technologies of the atomic force microscope (AFM) and microelectromechanical system (MEMS), is an emerging sensor technique. This technique utilizes the microcantilever bending, which is driven by the differential surface stress induced from the specific biomolecular interactions between target and probe molecules on a functional bimaterial microcantilever and recording of either optical or electronic signals. In comparison with enzyme-linked immunosorbent assays (ELISAs), microcantilever immunosensors have noticeable advantages of being label-free to detect multiple analytes in a single step in real time, in situ monitoring, and consequently lower the analytical cost. Since the 1990s, microcantilever immunosensors have been studied to detect various biomolecules and microorganisms, such as prostate-specific antigen, myoglobin, creatin kinase, bacterium Salmonella enterica, virus feline infectious peritonitis coronavirus, peptide, transcription factors SP1 and NF-κB, biomarker transcripts in human RNA, DNA hybridization, and the agrichemicals dichlorodiphenyltrichloroethane (DDT), 2,4-dichlorophenoxyacetic acid (2,4-D), and atrazine. Among the published microcantilever immunosensors for small molecules, only the atrazine antibodies were functionalized on the microcantilever, whereas the DDT and 2,4-D coating antigens were immobilized on the microcantilevers which requires the synthesis of hapten-protein conjugates and, thus, loses the advantage of microcantilevers.

It is known that the surface modification plays a critical role on developing a sensitive, reproducible, and reliable microcantilever immunosensor. Various reagents are used to immobilize the antibody or hapten–BSA conjugate on the gold (Au) side of the microcantilever surfaces. The reagents include the monothiol linkers 3-mercaptopropionic acid (2,4-D), atrazine. The concentration of clenbuterol in a fortified feed sample detected with the corresponding dcELISAs. The concentration of clenbuterol and the antibiotic chloramphenicol. In this paper, protein A showed to be simple and reproducible and purification agreed well with that detected with the dcELISA. Protein A showed to be simple and reproducible for functionalization of the antibodies on the Au surface and, thus, has common application values in microcantilever immunosensor development. The results suggest that microcantilever immunosensors be suitable for detection of small molecules, and the assay sensitivity is mainly related to the quality and activities of the antibodies.

on the microcantilever. This multilayer approach was found to involve polyethylenimine (PEI) based on the electrostatic attraction to bind with poly(sodium 4-styrenesulfonate) (PSS) and self-assembled monolayer (SAMS). The only disadvantage of IgG to interact freely with antigens. Several microcantilever sensors have been successfully established by using calixcrown for antibody orientation control. The homobifunctional cross-linkers DTSSP, DSU, sulfo-LC-SPDP, and SADA first react with the $-\text{NH}_2$ groups of the antibody to give an amide linkage, and then dithiothreitol (DTT) or hydroxylamine is used to reduce the disulfide to give a thiol for conjugation onto the Au surface. The sulphydrylated antibodies, however, lost a certain extent of activity due to possible breakage of the disulfide bonds between the heavy chain and light chain. Most polyclonal antibody molecules can be modified to contain up to six SADA molecules per IgG with minimal effects on their antigen binding activity. Some sensitive monoclonal antibodies, however, may be susceptible to modification and should be tested on a case-by-case basis. Unlike the monothiol and homobifunctional cross-linkers, calixcrown contains two SH groups for conjugation onto the Au surface; the crown moiety of the molecule has the ability to bind the ionized amine groups of the antibody without any activation reagent. Furthermore, the constant fragment (Fc) domain of IgG binds to calixcrown with vertical orientation, which allows the variable fragment (Fv) domain of IgG to interact freely with antigens. Several microcantilever sensors have been successfully established by using calixcrown self-assembled monolayer (SAMS). The only disadvantage of calixcrown is that it is expensive and not commercially available.

Another way to functionalize the gold side is a layer-by-layer multilayer self-assembly technique. The first monolayer was 2-mercaptoethanesulfonic acid (MCES) that the $-\text{SH}$ group was immobilized on the Au surface, and the $\text{SO}_3^-$ group was left free for interaction with poly(sodium 4-styrenesulfonate) (PSS) and polyethyleneimine (PEI) based on the electrostatic attraction to reach a multilayer on the microcantilever. The monoclonal antibody with a net negative charge at pH 7.0 was absorbed on the microcantilever. This multilayer approach was found much more reproducible than monothiol and homobifunctional cross-linkers for microcantilever surface modification, but the antibody immobilization protocols are very complicated.

Aminopropyltriethoxysilane (APTES) and the copoly(1-(3-aminopropyl)triethoxysilane–3-mercaptopropionic acid) (APTES–3MPA) were used to functionalize the silicon side of the microcantilever surfaces. APTES reacts with the Si–OH group of the silicon side via the active triethoxysilanol group of the molecule, and then the $-\text{NH}_2$ group on APTES reacts with succinic anhydride or glutaraldehyde to immobilize the antibody. Poorly reproducible response was observed from the microcantilevers, which indicates that the functionalization method is hard to produce an antibody monolayer film on the silicon side. The copoly(1-(3-aminopropyl)triethoxysilane–3-mercaptopropionic acid) (APTES–3MPA) obtained by radical copolymerization of $N,N$-dimethylethylacrylamide, $N$-acryloyloxy succinimimde, and $3$-(trimethoxysilyl)propyl methacrylate reacts with the Si–OH group of the silicon side via the active trimethoxysilanol side, and then the active esters side to immobilize the antibody. This is also a difficult functionalization method.

Clenbuterol (CL), one of synthesized $\beta$-adrenergic agonists, has been often used illegally as a growth-promoting agent to improve the efficiency of feed conversion and enhance lean meat to fat ratios in many species including cattle, sheep, swine, and humans. Chloramphenicol (CAP) is a broad-spectrum antibiotic widely used in veterinary practices as an economical and effective drug. However, because of serious toxic side effects such as acute poisoning of clenbuterol and dose-independent fatal aplastic anemia of chloramphenicol in humans, their usage in livestock is banned in many countries including China, the United States, and European countries. ELISA is now a main screening method for the determination of clenbuterol and chloramphenicol residues in edible animal tissues.

In the present study, protein A was used directly to absorb to the gold side of microcantilever, and the antibody activities on the microcantilever were detected with direct competitive ELISA (dcELISA). Microcantilever functionalization methods were investigated with two well-characterized polyclonal antibodies against clenbuterol and chloramphenicol. The microcantilevers developed were compared with the corresponding ELISAs.

**EXPERIMENTAL SECTION**

**Chemicals.** Clenbuterol, chloramphenicol, 2-iminothiolane hydrochloride, $3,3',5,5'$-tetrathoxymethylene (TMB), protein A (SPA), human serum albumin (HSA), bovine serum albumin (BSA), and horseradish peroxidase (HRP) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals that were of analytical grade were obtained from Beijing Chemical Reagents Co. (Beijing, China).

**Apparatus.** The 96-well polystyrene microtiter plates were purchased from Costar (Corning, NY). An automated plate-washer

---

(Wellwash 4 MK) and a microplate reader (Multiskan MK3) were purchased from Thermo (Vantaa, Finland). An electric heating constant-temperature incubator was purchased from Tianjin Zhonghuan Experiment Electric Stove Co. Ltd. (Tianjin, China). Silicon nitride microcantilevers (Veeco Instruments, Plainview, NY) were used. The dimensions of the V-shaped microcantilevers were 0.6 × 40 × 200 μm² (Figure 1). One side of the microcantilever had a thin film of chromium (15 nm) covered with a 60 nm layer of gold deposited by e-beam evaporation.

Buffers and Solutions. Buffers and solutions used include coating buffer (0.05 M carbonate buffer, pH 9.6), 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), citrate−phosphate buffer (0.01 M citric acid and 0.03 M monosodium phosphate, pH 5.5), substrate solution (4.0 mg of 30% hydrogen peroxide added to 10.0 mL of citrate−phosphate buffer containing 0.1 mg/mL TMB), and a stop solution (2.0 M sulfuric acid).

Immunoreagents Preparation. HSA-CL and HRP-CL, used as immunogen and enzyme conjugate, respectively, were synthesized via the diazotization method described previously. BSA-CAP and HRP-CAP, used as immunogen and enzyme conjugate, respectively, were synthesized with the hapten chloramphenicol succinate via the activated ester method described by Kolosova et al. The anti-CL polyclonal antibody (pAb) and anti-CAP polyclonal antibody were available from our previous studies, which were purified from corresponding antisera by ammonium sulfate precipitation followed by a protein A column purification (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer’s manual.

Antibody Sulphydrylation. To 1.0 mL of 10 mg/mL anti-CL pAb PBS was added 45.8 μL of 2.0 mg/mL freshly prepared 2-iminothiolane solution in distilled water. After approximately 1 h at room temperature, the reaction mixture was dialyzed against 20 mM PBS buffer (containing 150 mM NaCl and 1.0 mM EDTA, pH 7.2) for 48 h. The diazylated sulphydrated PAb was diluted to 1.0 mg/mL and stored at −40 °C. The activities of the antibodies before and after sulphydrlation were tested by dcELISA.

Microcantilever Functionalization. Functionalization with Protein A. The functionalization procedures on the microcantilever were all in the microtiter well that was replaced to a new one after each step. Each microcantilever was pretreated with “piranha dip” (H₂O₂/H₂SO₄ = 1:3, 100 μL) for 30 min and washed with deionized water (200 μL) for three times. The microcantilever was dried under a gentle stream of nitrogen gas. An aliquot of 200 μL of 10 μg/mL SPA coating buffer was pipetted into the well followed by incubation for 3 h at 37 °C. The microcantilever was washed with PBST for 3 times, 200 μL of 3% nonfat dry milk coating buffer was then added followed by incubation for 30 min at 37 °C for blocking of the silicon nitride side. After the microcantilever was washed with PBST for three times, it was immersed into the 200 μL of 4.0 μg/mL anti-CL antibodies solution diluted by PBSTG and incubated for about 3 h at 37 °C. The anti-CL antibodies coated microcantilevers, referred to as functionalized microcantilevers, were washed with PBST for three times and dried again, which were ready to use.

The process of functionalized microcantilevers with anti-CAP antibodies was the same as that of anti-CL antibodies coated microcantilevers.

Functionalization with 2-Iminothiolane Sulphydrated Antibodies. The functionalization procedures on the microcantilever were all in the microtiter well that was replaced to a new one after each step. The washed and blocked microcantilever was immersed in 200 μL of 4.0 μg/mL 2-iminothiolane sulphydrated anti-CL antibodies solution diluted by PBSTG and incubated for about 3 h at 37 °C. The 2-iminothiolane sulphydrated anti-CL antibodies coated microcantilevers were washed with PBST for three times and dried; the functionalized microcantilevers were ready to use.

Characterization of Inhibition of the Antibody Immobilized on the Microcantilever Using dcELISA. The enzyme immunosassay on the microcantilever was all in the microtiter well that was replaced to a new one after each step. One of the functionalized microcantilevers was put into a microtiter well that was replaced to a new one after each step. One of the functionalized microcantilevers was the same as that of anti-CL antibodies coated microcantilevers.

Analytical Chemistry, Vol. 82, No. 2, January 15, 2010 617
temperature was maintained at 301 ± 0.5 K. In the protein A functionalized microcantilever immunosensor, clenbuterol and chloramphenicol were added into the fluid cells at a concentration range from 0.1 to 10.0 ng/mL and from 0.2 to 1000 ng/mL, respectively, followed by microcantilever deflection measurement.

dcELISA. The concentrations of the coating anti-CL antibody and HRP-CL (1.0 mg/mL) were 4.0 µg/mL in coating buffer and 1:4000 dilution in PBSTG, respectively. The concentrations of the coating anti-CAP antibody and HRP-CAP (1.0 mg/mL) were 2.0 µg/mL in coating buffer and 1:8000 in PBSTG, respectively. After the procedures of coating, competition, color reaction, and absorbance reading at 405 nm were completed, the concentrations of the analytes were calculated with the ORIGIN 7.5 analysis software and the concentrations causing 20% inhibition (IC_{20}) were estimated as the limit of detection.

Preparation and Extraction of Sample. A control feed sample was purchased from a local market in Beijing. The sample was extracted and cleaned up in triplicate according to the procedure as described by He et al. Briefly, 5.0 g of powdery sample was blended and fortified with 100 µL of 250 ng/mL clenbuterol standard. The fortified sample was extracted with 2 × 15 mL of 0.1 M perchloric acid. After the extracts were combined, the pH value was adjusted to 10 with 30% ammonium hydroxide. Liquid–liquid extractions were performed with 2 × 15 mL of ethyl acetate and isopropyl alcohol (9:1, v/v). The organic phases were combined and were then evaporated to dryness at 50 °C under a gentle stream of N₂ gas. Finally, the residue was reconstituted with 5 mL of 0.02 M ammonium acetate buffer solution (pH 5.2) and was subjected to solid-phase extraction (SPE) cleanup.

The extracts were loaded onto an SCX column previously conditioned with 5 mL of methanol, 5 mL of water, and 5 mL of 0.03 M hydrochloric acid. The flow rate was less than 1 mL/min. The cartridges were washed sequentially with 5 mL of water and 5 mL of methanol. Subsequently, the column was dried and the target chemical was eluted with 5 mL of ammonium hydroxide in methanol (3%, v/v). Finally, the elution solution was dried at 50 °C under a gentle stream of N₂ gas and redissolved with 5.0 mL of PBS.

RESULTS AND DISCUSSION
Inhibitory Activities of Antibodies Functionalized on the Microcantilever. Protein A and protein G can specifically bind the Fc region of an antibody. Unlike other immobilization reagents such as monothiol and homobifunctional cross-linkers, protein A and protein G can site-specifically immobilize antibody on a solid surface without chemical reaction, which preserves the biological activity and enhances antigen detection. Genetically engineered cysteine-tagged protein G has been used for antibody immobilization through the thiol group adsorption on the gold surface. Surface plasmon resonance (SPR) and SPR imaging analyses indicated that a gold surface treated with cysteine-tagged protein G possesses a superior antibody binding ability compared to tag-free protein G. Protein A has been used as a linker for antibody immobilization in the piezoelectric crystal immunosensor.

<table>
<thead>
<tr>
<th>concentration of clenbuterol (ng/mL)</th>
<th>absorbance ± SD</th>
<th>relative antibody activity changes (%)</th>
<th>absorbance ± SD</th>
<th>relative antibody activity changes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.428 ± 0.025</td>
<td>1.275 ± 0.043</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1.066 ± 0.018</td>
<td>0.925 ± 0.031</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>0.498 ± 0.009</td>
<td>0.443 ± 0.016</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>50.0</td>
<td>0.114 ± 0.003</td>
<td>0.106 ± 0.004</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

* The absorbance values (±SD, n = 5) showed the loss of the antibody activity due to sulfhydrylation with 2-iminothiolane relative to the native antibody in the competitive direct ELISA. The concentrations of the native and sulfhydrylated anti-CL antibodies were 4.0 µg/mL.

va et al. employed two sequential reactions of using APTES and glutaraldehyde to activate the silicon side of the microcantilevers on which protein A is immobilized. Such an immobilized protein A layer defines the orientation of the antibody to remain fully functional, potentially improving the sensitivity of the biosensor.

In the present study, protein A was used to directly functionalize the gold surface of the microcantilever. It is thought that the major driving force in the protein A binding on a gold surface is the N–Au interaction, but the strength was reported to be much weaker than the S–Au bonding. Because microcantilevers were not regenerated for reuse in the present study, the effect of interaction force between protein A and Au was not considered in the study. In addition to protein A, 2-iminothiolane was used to introduce thiol groups on the antibody for microcantilever functionalization. The advantage is that the thiol group can be introduced to the antibody in a single step and need not add hydroxylamine to deprotect the acetylated thiol of the SATA-modified antibody or DTT to reduce the disulfide group, which disulfides indigenous to the native antibody will not be affected. The antibody activity change after sulfhydrylation was not well-described in the literature. The dcELISA results showed that sulfhydrylation of the anti-CL antibody by using 2-iminothiolane caused a loss of 7–12% of the binding activities (Table 1).

A number of techniques, including contact angle, infrared, ellipsometry, circular dichroism spectroscopy, AFM, and scanning electron microscopy, were used to confirm the antibodies state on the gold surface of the microcantilever. Grogan et al. used enzyme- or fluorescence-labeled secondary antibodies to estimate the immunological activity and density of the antibody functionalized on the microcantilever. Such a method relies on the recognition of the immobilized antibody by the labeled secondary antibody to indirectly estimate the activity and density of the antibody rather than direct measurement of the actual inhibitory activity of the antibody functionalized on the gold side.

The activity of the sulfhydrylated anti-CL antibody immobilized on the microcantilever was compared with that immobilized via protein A. When the microcantilevers functionalized via 2-iminothiolane were placed in microplate wells to determine the activity of the sulfhydrylated anti-CL antibody, the absorbance values of the clenbuterol-free and clenbuterol-present wells were 0.425 ± 0.019 and 0.079 ± 0.002, respectively (Figure 2). The microcantilevers functionalized via protein A were placed in


<p>| Table 1. Comparison of Binding Activities of the Anti-CL Antibodies Before and After Sulfhydrylation |
|---------------------------------------------------------------|-----------------------------------------------|-----------------|</p>
<table>
<thead>
<tr>
<th>concentration of clenbuterol (ng/mL)</th>
<th>absorbance ± SD</th>
<th>relative antibody activity changes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.428 ± 0.025</td>
<td>1.275 ± 0.043</td>
</tr>
<tr>
<td>0.5</td>
<td>1.066 ± 0.018</td>
<td>0.925 ± 0.031</td>
</tr>
<tr>
<td>5.0</td>
<td>0.498 ± 0.009</td>
<td>0.443 ± 0.016</td>
</tr>
<tr>
<td>50.0</td>
<td>0.114 ± 0.003</td>
<td>0.106 ± 0.004</td>
</tr>
</tbody>
</table>
microplate wells to determine the activity of the antibody, the absorbance values of the clenbuterol-free and clenbuterol-present wells were 0.737 ± 0.024 and 0.081 ± 0.004, respectively (Figure 2). Although both functionalization methods were suitable, protein A immobilization of the antibody preserved the antibody activities better than the direct sulfhydrylation immobilization (Figure 2). The decreased activity of the sulfhydrated anti-CL antibody may be attributed to disulfide bond breakage and denaturation of the anti-CL antibody during the sulfhydrylation process. Immobilized protein A probably allows proper orientation of the antibodies to remain at their full activity. The protein A functionalized microcantilevers were thus used in the remainder of the studies.

**Microcantilever Immunosensor Detection.** Nonspecific adsorption of antibodies onto the silicon nitride side of the microcantilevers should be avoided when establishing a new microcantilever immunosensor. It is reported that the silicon nitride side is inert to nonspecific attachment of antibodies by formation of a poly(ethylene glycol) (PEG)—silane-grafted silicon surface. The PEG—silane treatment can significantly reduce protein physicoadsorption. Fragment BSA and casein are also used to minimize nonspecific adsorption of antibodies onto the silicon nitride side of the microcantilevers. In the present study, 3% nonfat dry milk in coating buffer was used to block the microcantilevers prior to addition of polyclonal antibody to functionalize microcantilevers.

Watari et al. reported that the absolute cantilever bending can arise from specific and nonspecific effects. In situ reference cantilevers were found to be useful to probe specific bending signals. In the present study, although one microcantilever was employed to detect the bending, control microcantilevers were used to in separate runs to cancel the nonspecific effects such as buffer pH, ionic strength, and nonspecific interferences. It is noteworthy that in preliminary studies various small organic compounds including 1-naphthaleneacetic acid, choline chloride, parathion-ethyl, chlorimuron-ethyl, salbutamol, chloramphenicol, and clenbuterol up to a concentration of 1000 ng/mL showed negligible nonspecific deflection to the naked microcantilevers (data not shown). The nonspecific deflections of clenbuterol and chloramphenicol to the naked microcantilevers are shown in Figure 3, parts A and B, respectively. When the microcantilevers functionalized with clenbuterol antibody using the protein A method were exposed to 1000 ng/mL of chloramphenicol or the microcantilevers functionalized with chloramphenicol antibody using the protein A method were exposed to 1000 ng/mL of clenbuterol, no significant response was detected, indicating little interferences from nonspecific small molecules on the microcantilever immunosensor (Figure 3A).

Figure 3 shows the relation between the concentration of clenbuterol and the deflection of microcantilever. When the limit of detection (LOD) is defined as a signal of 3-fold of the background noise, the results showed that the LOD of microcantilever immunosensor for clenbuterol is approximately 0.1 ng/mL.

A pig feed sample fortified with clenbuterol at 5.0 ng/g was thoroughly extracted, cleaned up, and then was detected with the microcantilever immunosensors as well as dCELSA. The concen-
tation of clenbuterol detected with the microcantilever was approximately 4.4 ng/g (Figure 3A), and it was 4.65 ng/g with dcELISA. The average recoveries of clenbuterol determined with the microcantilever and dcELISA were 88% and 93%, respectively.

To demonstrate uses of microcantilever immunosensors in which protein A is used as the functionalization reagent for other small molecules, a microcantilever immunosensor for chloramphenicol was developed according to the same procedure as the above-described clenbuterol microcantilever (Figure 3B). The results showed that the LOD of the microcantilever immunosensor for chloramphenicol was approximately 0.2 ng/mL.

Although the LODs of the microcantilever immunosensor for clenbuterol and chloramphenicol were similar, the binding kinetics between the analyte and the corresponding antibody immobilized on microcantilevers via protein A differed largely. The deflections of microcantilevers for clenbuterol and chloramphenicol were 51 and 24 nm, respectively; the former is about 5 times larger than the latter. When the concentration was 10 ng/mL, the deflections of microcantilever for clenbuterol and chloramphenicol were 247 and 47 nm, respectively; the former was approximately 2 times larger than the latter. The binding capability of the anti-CL antibody was significantly higher than that of the anti-CAP antibody, particularly at low concentrations of analytes. This may be attributed to differences in the dissociation constants ($K_d$) of the two antibodies. The $K_d$ values of anti-CL antibody and anti-CAP antibody were $3.5 \times 10^{-10}$ and $2.4 \times 10^{-9}$ M, respectively.

Limits of Detection of Microcantilever Immunosensor and dcELISA. Wu et al.\textsuperscript{1} reported that longer microcantilevers produced larger deflections under the same concentration of the analyte, thereby having higher sensitivity. Other factors affecting the immunosensor sensitivity include the affinity and specificity of the antibody functionalized on the microcantilever and functionalization methods. In the present study, the LODs of clenbuterol and chloramphenicol were 0.1 and 0.2 ng/mL, respectively, when a 200 µm long and 0.6 µm thick silicon nitride microcantilever was used (Figure 3). The dcELISAs of clenbuterol and chloramphenicol had the LODs of 0.2 and 1.5 ng/mL, respectively (Figure 4). The LODs of the microcantilever immunosensors for clenbuterol and chloramphenicol were lower than those of the corresponding dcELISA. Suri et al.\textsuperscript{12} reported an atrazine microcantilever immunosensor with an LOD of 4.65 pM (0.001 ng/mL) which was approximately 10 times lower than that of the competitive ELISA (0.01 ng/mL). The microcantilever immunosensors for clenbuterol and chloramphenicol, however, require approximately 4–8 h of equilibrium time upon varying concentrations of the analytes. In the present study, the microcantilevers were not regenerated for reuse, which requires a high degree of uniformity of physical features of the microcantilevers. Regeneration of functionalized microcantilevers for reuse may provide more consistent data because it could cancel variations among microcantilevers, which requires further investigation on changes of activity and density of the antibody functionalized on the regenerated microcantilevers.

CONCLUSIONS

Protein A was used to functionalize the gold surface of the microcantilevers, and the functionalized microcantilevers showed good repeatability and dynamic range of the detection. A simply and direct ELISA was employed for validation of the antibody functionalization on the microcantilever Au surface. The LODs of the microcantilever immunosensors were approximately 0.1 and 0.2 ng/mL of clenbuterol and chloramphenicol, respectively, which were better than those of the corresponding dcELISA. Analyses of the fortified samples suggest that the results by the microcantilever agreed well with those by the dcELISA. The results suggest that protein A is suitable to functionalize microcantilevers for small molecules. The sensitivity, however, would mainly depend upon microcantilever dimension, shape, and the affinity and specificity of the antibodies.

ACKNOWLEDGMENT

This work was supported in part by the National Natural Science Foundation of China (Grant Nos. 10732080, 10627201, and 10872191) and the National Basic Research Program of China (2006CB300404). We thank Dr. Qing X. Li in the University of Hawaii for helpful discussion and careful review of this manuscript. The first two authors contributed equally to this work.

Received for review August 27, 2009. Accepted November 20, 2009.