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Development of a Secondary Antibody Thio-Functionalized Microcantilever Immunosensor and an ELISA for Measuring Ginsenoside Re Content in the Herb Ginseng

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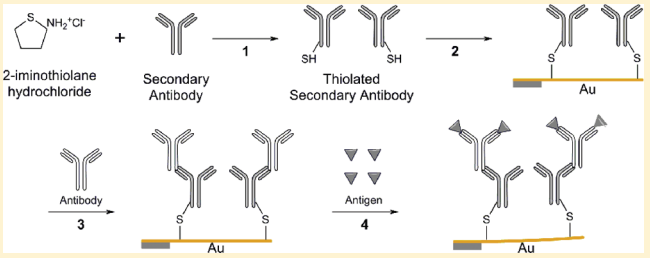
ABSTRACT: Ginsenoside Re (GRe) is a major active component of the Chinese medicinal herb ginseng, *Panax ginseng*. A sensitive and specific monoclonal antibody (mAb), designated as mAb3D6, was generated with a GRe−bovine serum albumin conjugate as an immunogen. Microcantilever immunosensors (MCS), one modified with thiolated anti-GRe antibody and one modified with thiolated goat antimouse immunoglobulin G (IgG), were developed to detect the content of ginsenoside. The MCS immobilized with thiolated goat antimouse IgG had a better sensitivity than the MCS modified with thiolated anti-GRe antibody. The advantage of a secondary antibody thio-functionalized MCS was verified with the anti-paclitaxel mAb. An indirect competitive enzyme-linked immunosorbent assay (icELISA) was also established with mAb3D6. The concentration of analyte producing 50% inhibition and the working range of icELISA were 1.20 and 0.15−16.1 ng/mL, respectively. The icELISA had a cross-reactivity of 89% with ginsenoside Rg1 and less than 3% with other ginsenosides. The icELISA and MCS with thiolated secondary antibody were applied for the determination of GRe in ginseng samples, and the results agreed well with those determined by high-performance liquid chromatography.



Ginseng (*Panax ginseng*) has been one of the most popular medicinal herbs in China for thousands of years. It is used to improve the cardiovascular and endocrine systems, enhance immunity, and promote metabolism, antitumor activity, and antioxidation.[1−4] Ginsenosides (Table 1), the major bioactive component and a quality control marker of ginseng, are mainly found in the ginseng roots. To date, more than 40 ginsenosides have been identified.[5] Ginsenoside Re (GRe) is the major component in the American ginseng (*Panax quinqefolium*).[6] GRe has antidiabetic, antioxidant, and anti-ischemic effects and increases the proliferation of CD^{4+} T cells.[6−9] The content of GRe in the *P. ginseng* root varies considerably with the cultivar, cultivation region, climatic conditions, and harvest age of the ginseng roots. Illicit trades in counterfeit and substandard ginseng were found in the Chinese herbal medicine market.[10] To screen a large number of wild and cultured, true and false *P. ginseng* root samples for quality control, a sensitive and specific analytical method for GRe is needed.

A number of methods have been developed for the detection of ginsenosides, including thin-layer chromatography (TLC),[11,12] high-performance liquid chromatography (HPLC),[13−16] gas chromatography/mass spectrometry (GC/MS),[17] HPLC−MS,[18−21] and HPLC−tandem mass spectrometry.[22−24] Among these methods, the TLC method with low sensitivity and resolution is not suitable for quantitative detection of ginsenosides. The other instrumental methods are expensive and time-consuming and need rigorous sample preparation. An enzyme-linked immunosorbent assay (ELISA) was reported for the analysis of 20(*S*)-protopanaxatriol[25] and GRg1.[25,26] A chromatographic immunostaining method was reported for the detection of GRe.[6,27] ELISA is rapid, cost-effective, selective, and sensitive and has been used in the detection of plant secondary metabolites.[28]

The microcantilever immunosensor (MCS) is an emerging sensor technique utilizing the bending response of the microcantilever, which is driven by the changes in surface stress induced by the interaction of antibody and antigen, and recording of either optical or electronic signals.[29] Compared with ELISA, the MCS has notable advantages of being label-free to detect multiple analytes in a single step in real time, in situ monitoring.

Since the 1990s, MCSs have been studied for the detection of various biomacromolecues and small molecules.[30] It is known that surface modification contributes significantly to MCS sensitivity and reproducibility. Various reagents such as 2-iminothiolane hydrochloride,[31−33] monothiol linkers,[34−36] cysteamine,[37] cystamine dehydrochloride,[38] aminoethane-

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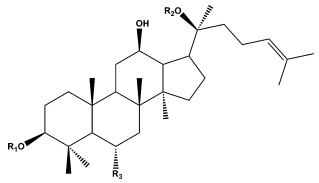
Table 1. Cross-Reactivity of GRe and Related Analytes



analyte	IC_{50} (ng/mL)	cross-reactivity[a] (%)	R1	R2	R3
GRe	1.20 ± 0.06[b]	100.0	−H	−Glc[c]	−OGlc-^{2}Rha
GRg1	1.34 ± 0.11	89.6 ± 7.9	−H	−Glc	−OGlc
GRd	43.53 ± 3.40	2.8 ± 0.2	−Glc-^{2}Glc	−Glc	−H
GRf	46.14 ± 4.19	2.6 ± 0.2	−H	−H	−OGlc-^{2}Glc
GRb1	>2000	<0.1	-Glc-^{2}Glc	−Glc-^{6}Glc	−H
GRc	>2000	<0.1	−Glc-^{2}Glc	−Glc-^{6}Ara(f)	−H

[a]Cross-reactivity (%) = (IC_{50} of GRe/IC_{50} of other compound) × 100. [b]The data represent the mean of triplicate analyses ± SD. [c]Abbreviations: Glc, β-D-glucopyranosyl; Rha, α-L-rhamnopyranosyl; Ara(f), α-L-arabinofuranose.

thiol,[39] homobifunctional cross-linkers,[40,41] dextran,[42] calix crown,[43,44] staphylococcal protein A (SPA),[31] and genetically engineered cysteine-tagged protein G[45] were used to immobilize the antibody or hapten−bovine serum albumin (BSA) conjugate on microcantilever Au surfaces. 2-Iminothiolane hydrochloride can react with a primary amine to create a sulfhydryl group and thiolate the antibody efficiently and rapidly. Cysteamine, cystamine dehydrochloride, aminoethanethiol, monothiol linkers, and homobifunctional cross-linkers undergo chemical reaction to immobilize antibody. These methods may decrease the antibody activities, and it is difficult to control a proper antibody orientation. Dextran can immobilize much more antibody through a unique spatial configuration, but it also needs activation reagents such as ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) and has no control on antibody orientation. Protein A can site-specifically immobilize antibody on a solid surface, which preserves the biological activity and enhances antigen detection, but the major driving force in the protein A binding on a gold surface (N−Au interaction) is much weaker than S−Au bonding. Calix crown and genetically engineered cysteine-tagged protein G can immobilize antibody without chemical reaction and ensure a good orientation control, but the two reagents are expensive and not commercially available.

In the present study, a monoclonal antibody (mAb) against GRe was produced and used to develop a highly sensitive indirect competitive ELISA (icELISA). An MCS immobilized with thiolated anti-GRe and an MCS immobilized with thiolated goat antimouse immunoglobulin G (IgG) were developed, and the sensitivity of the two MCSs were compared. The icELISA and the secondary antibody thio-functionalized MCS were applied for the determination of GRe in ginseng samples, and the results were confirmed by high-performance liquid chromatography.

EXPERIMENTAL SECTION

Chemicals. Ginsenoside Re (GRe), ginsenoside Rg1 (GRg1), ginsenoside Rb1 (GRb1), ginsenoside Rc (GRc), ginsenoside Rd (GRd), and ginsenoside Rf (GRf) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Anti-paclitaxel mAb was generated in our laboratory.[46] Bovine serum albumin (BSA), ovalbumin (OVA), goat antimouse IgG, 2-iminothiolane hydrochloride, 3,3′,5,5′-tetramethylbenzidine (TMB), and phosphoric acid of chromatography grade were purchased from Sigma-Aldrich (St. Louis, MO). Goat antimouse IgG conjugated with horseradish peroxidase (IgG−HRP) was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Cell culture medium (Dulbecco's modified Eagle's medium, DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (Paisley, Scotland). Acetonitrile of chromatography grade was purchased from Fisher Scientific (New Jersey). All other reagents were obtained from Beijing Chemical Reagents Co. (Beijing, China).

Apparatus. Cell culture plates and 96-well polystyrene microplates were purchased from Costar (Corning, NY). The electric heating constant-temperature incubator was purchased from Shanghai Zhicheng Analytical Instrument Manufacturing Co. Ltd. (Shanghai, China). The automated plate washer (Wellwash 4 MK2), microplate reader (Multiskan MK3), and direct heat CO_2 incubator were purchased from Thermo (Vantaa, Finland). The Agilent 1200 HPLC system consists of a quaternary pump and a VWD detector (Agilent Technologies, Santa Clara, CA). The 0.2 and 0.45 μm syringe filters were purchased from Pall (Ann Arbor, MI). Silicon nitride V-shaped microcantilevers with dimensions of 0.6 × 40 × 200 μm (Veeco Instruments, Plainview, NY) were used. 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. The 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 urea peroxide added to 10.0 mL of citrate−phosphate buffer containing 0.1 mg/mL TMB), and a stop solution (1.0 M hydrochloric acid). Deionized water used for making buffers and solutions was collected from a Millipore water purification system (Millipore Co., Billerica, MA).

Preparation of Protein−Hapten Conjugates. GRe (Table 1) was conjugated to BSA and OVA as an immunogen and a coating antigen, respectively. The protein conjugates were synthesized via a periodate oxidation method as previously described with slight modification.[25] To a solution of 10 mg of GRe dissolved in 2 mL of methanol was added dropwise 1 mL of 0.01 M sodium periodate solution. The solution was stirred for 20 min at room temperature followed by addition of 60 μL

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of 0.1 M aqueous ethylene glycol and the resulting solution stirred for another 10 min. The reaction mixture was added dropwise to 50 mg of BSA or OVA dissolved in 5 mL of 0.05 M carbonate buffer (pH 9.6), and the pH value was adjusted to 9.3 with 0.1 M K_2CO_3. After 1 h, 1 mL of a fresh solution of KBH_4 (2.82 mg) in H_2O was added dropwise, and the whole mixture was further stirred for 1 h at 4 °C; then the pH value was adjusted to 6.5 with 0.1 M HCl and the mixture stirred for another 1 h. The mixture was dialyzed against 3 L of 0.01 M phosphate buffer containing 0.15 M NaCl (PBS, pH 7.5) for 3 days with two changes per day, then lyophilized, and stored at −20 °C.

Immunization Protocol and Monoclonal Antibody Production, Purification, and Characterization. Five female Balb/c mice, 6 weeks old, were immunized with the immunogen (GRe−BSA). The protocols of immunization, fusion, and antibody production and purification were the same as described previously.[47] The specificity of the mAb's was evaluated by cross-reactivity with ginsenosides (Table 1) utilizing icELISA.

Indirect Competitive ELISA. The protocol for icELISA was the same as that described previously.[47] A microplate was coated with 100 μL of 0.06 μg/mL GRe−BSA in coating buffer for 3 h at 37 °C. After being washed with PBST four times, the plate was blocked with 100 μL per well of 3% nonfat milk in PBS for 30 min at 37 °C and then was washed with PBST four times. To the microplate was added 50 μL per well of standard sample or analytes in PBSTG, followed by 50 μL of mAb in PBSTG. After incubation at 37 °C for 30 min, the plate was washed again with PBST four times, and then an aliquot of 100 μL per well of goat antimouse IgG−HRP diluted in PBSTG was added. After incubation at 37 °C for 30 min, the plate was washed again with PBST four times, and then 100 μL per well of substrate solution was pipetted. Finally, the reaction was terminated by adding 100 μL of the stop solution per well, and the absorbance at 450 nm was read with the microplate reader. The detection data of each analyte were calculated by using the Origin Pro 7.5 software.

Preparation and Extraction of Samples. Samples of *P. ginseng* were harvested from Jilin Province in China. All samples were dried in an electric heating constant-temperature incubator before being finely powdered. In this study, the hairy root of *P. ginseng* was used as the analytical sample. Ginseng samples were extracted according to the method previously described.[27] Briefly, the dried samples of various ginseng (100 mg) hairy roots were extracted with 2 mL of methanol under sonication five times and centrifuged at 7000*g* for 2 min. The supernatant was filtered, divided into two equal aliquots, and then evaporated for use. One resultant residue was dissolved in 4.0 mL of PBS and detected in the icELISA after dilution with PBS. The dilution ratio was 50 000 and 4000 for icELISA and MCS, respectively. The other resultant residue was dissolved in 4.0 mL of a mixture of H_2O−CH_3CN containing 0.5% H_3PO_4 (80:20, v/v) and filtered with a 0.45 μm Millipore membrane. The filtered solution was used for HPLC analysis.

Recovery Test for GRe Spiked in *P. ginseng* Samples. Samples (50 mg) of *P. ginseng* hairy roots, the GRe contents of which were quantified by icELISA, were spiked with GRe at concentrations ranging from 2.5 to 40 mg/g. The sample of *P. ginseng* hairy roots with no GRe added was used as the blank control. After being kept at 4 °C overnight, the samples were extracted according to the extraction procedure for ELISA and MCS as described in the previous section. The extracts were then analyzed with icELISA. Three separate extracts were taken for each spiked sample, and each extract was analyzed in triplicate.

HPLC Analysis of GRe. Standards and the samples of *P. ginseng* hairy roots were analyzed with HPLC according to the procedure previously described.[25] A Zorbax Eclipse XDB C18 column (150 × 4.6 mm, 5 μm particle size) (Agilent Technologies, Santa Clara, CA) was used to separate GRe. The mobile phase was a mixture of H_2O−CH_3CN containing 0.5% H_3PO_4 (80:20, v/v) at a flow rate of 1 mL/min. The UV absorption was detected at 202 nm. The injection volume was 10 μL. The retention time of GRe was approximately 20 min. The mobile phase, standards, and sample extracts obtained above were filtered through a 0.45 μm filter prior to HPLC. All data were collected and analyzed by an Agilent Chemstation.

Anti-GRe Antibody and Goat Antimouse IgG Sulfhydrylation. The sulfhydrylation protocol was the same as previously described.[31] The dialyzed sulfhydrylated anti-GRe antibody and sulfhydrylated goat antimouse IgG were diluted to 1.0 mg/mL with PBS and stored at −40 °C.

Functionalization of Microcantilevers. The functionalization was performed in the microplate well that was replaced with a new one after each step. The microcantilever was immersed in washing solution (3 mL of sulfuric acid and 1 mL of 30% H_2O_2) for 10 min before use and then was washed with deionized water three times. A 100 μL volume 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.

Immobilization via Thiolated Anti-GRe Antibody. After being washed three times with PBST, the microcantilever was immersed into 100 μL of 4.0 μg/mL sulfhydrylated antibody solution diluted with PBSTG and incubated for 2 h at 37 °C. The sulfhydrylated anti-GRe antibody-coated microcantilever was washed with PBST three times and dried under a gentle stream of nitrogen gas for use. Another microcantilever was immersed in 100 μL of 2.5 μg/mL unsulfhydrylated anti-GRe antibody solution in PBSTG, which was applied to check the functionalized reagent.

Immobilization via Thiolated Goat Antimouse IgG and Then Coupling with Anti-GRe Antibody. After the microcantilever was washed with PBST three times, the microcantilever was immersed in 100 μL of 2.5 μg/mL sulfhydrylated goat antimouse IgG solution in PBSTG for 30 min at 37 °C. The microcantilever was washed with PBST three times and then immersed into 100 μL of 0.12 μg/mL anti-GRe mAb solution diluted by PBSTG and incubated for about 3 h at 37 °C. The anti-GRe mAb-coated microcantilever, referred to as the functionalized microcantilever, was washed with PBST three times and dried under a gentle stream of nitrogen gas for use. Another microcantilever was immersed in 100 μL of 2.5 μg/mL unsulfhydrylated goat antimouse IgG solution in PBSTG, which served as a control.

Characterization of the Activity of the mAb Immobilized on the Microcantilever Using ELISA. ELISA was used to confirm the antibodies were immobilized on the microcantilever. The functionalized microcantilevers and the control microcantilevers were put in microplate wells. To the wells was added 100 μL of goat antimouse IgG−HRP diluted in PBSTG. After incubation at 37 °C for 30 min, the microcantilever was washed four times with PBST. After the microcantilever was transferred to a new well, 100 μL per well of substrate solution was added followed by incubation at 37 °C for 15 min. The

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reaction was stopped by adding 100 μL of the stop solution. The microcantilever was removed from the well, and the absorbance was read at 450 nm on a microplate reader.

MCS Detection. The functionalized microcantilever was mounted onto the fluid cell filled with PBS. The PBS was circulated through the cell by using a syringe pump. The flow rate was controlled at 4 mL/h and kept constant during each experiment. The temperature of the fluid cell was controlled at 310 ± 0.01 K while the room temperature was maintained at 301 ± 0.5 K.

MCS Detection with Thiolated Anti-GRe Antibody. After the deflection baseline was stabilized, the analyte GRe dissolved in PBS was injected into the fluid cell at a concentration range from 10 to 1000 ng/mL, followed by microcantilever deflection measurement.

MCS Detection with Thiolated Goat Antimouse IgG and Then Coupled with Anti-GRe Antibody. After the deflection baseline was stabilized, GRe dissolved in PBS was injected into the fluid cell at a concentration range from 0.5 to 1000 ng/mL, followed by microcantilever deflection measurement.

Versatility of the Secondary Antibody Thio-Functionalized MCS. The MCS was functionalized with thiolated secondary antibody and then coupled with anti-paclitaxel antibody. Paclitaxel is one of the most effective natural anticancer products extracted from the yew plant, and it is widely used in the medical treatment of ovarian, breast, and non-small-cell lung cancer.[46] The treatment of the functionalized microcantilever was the same as above. The analyte paclitaxel dissolved in PBS was injected into the fluid cell at a concentration range from 1.0 to 50 ng/mL, followed by microcantilever deflection measurement.

The detection data of each analyte were calculated by using the Origin Pro 7.5 software. A double logarithm calibration curve was made between the deflection responses and the varying concentrations of standard samples in PBS, and the concentrations of analyte in the samples were calculated by using the calibration curve.

RESULTS AND DISCUSSION

Characteristics of mAb against the GRe−BSA Conjugate. The GRe molecule has a reactive functional group that makes it easy to covalently link to a protein carrier directly. Morinaga et al.[27] synthesized the GRe−BSA conjugate with a modification of the procedure of Erlanger and Beiser.[48] In the present study, we prepared the immunogen and coating antigen via a periodate oxidation method;[25] the conjugate treated with KBH_4 possesses a more stable structure than Morinaga's GRe−BSA conjugate.

A monoclonal antibody, designated as mAb3D6, was generated with GRe−BSA as the immunogen. The dissociation constant (K_d) of mAb3D6 was determined with the method of Beatty.[49] The K_d value was 6.7×10^{-10} M. mAb3D6 is an IgG1 isotype that has κ light chains. The cross-reactivity of the icELISA with GRg1, GRd, and GRf is 90%, 2.8%, and 2.6%, respectively. The assay has a cross-reactivity of less than 0.1% with GRb1 and GRc (Table 1).

Optimization of icELISA. The optimal concentrations of coating antigen, mAb, and goat antimouse IgG−HRP were screened by checkerboard titration. Concentrations of 0.06 μg/mL GRe−BSA, 0.12 μg/mL mAb3D6, and 0.1 μg/mL goat antimouse IgG−HRP were selected and used throughout this work. Figure 1 shows a representative inhibition curve for GRe by icELISA. The IC_{50} value of the icELISA was approximately

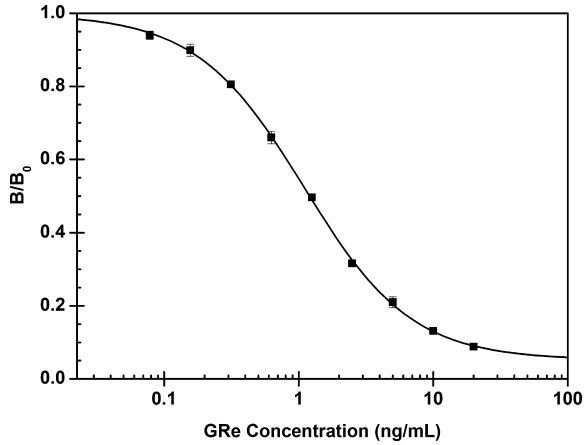

Figure 1. Standard inhibition curve of GRe by icELISA. B_0 and B are absorbances in the absence and presence of competitors, respectively. Concentrations causing 50% and 10% inhibition by GRe were 1.20 and 0.15 ng/mL, respectively.

1.20 ng/mL, and the calibration range, based on 10−90% inhibition of binding of mAb3D6 to GRe, was 0.15−16.1 ng/mL.

Recovery of GRe from Fortified *P. ginseng* Samples by icELISA. The average recoveries for 2.5−40 mg/g spiked GRe ranged from 94.0% to 97.4%, and the overall average was 96.0% (Table 2).

Table 2. Recovery of GRe Spiked to *P. ginseng* Samples

GRe concn (mg/g)		
spiked	detected[a]	mean recovery (%, n = 3)
0	10.46	0
2.5	12.81	94.0 ± 0.31[b]
5	15.33	97.4 ± 0.45
10	20.01	95.5 ± 0.82
20	29.89	97.2 ± 1.46
40	48.86	96.0 ± 1.71

[a]The data are the means of triplicate samples. Recoveries were determined after subtraction of the background concentration of GRe. [b]The data represent the mean ± SD.

Analysis of GRe in *P. ginseng* Samples with icELISA and HPLC. The GRe content in five *P. ginseng* hairy root samples with different growing years was determined with icELISA and HPLC. Morinaga et al.[27] developed a chromatographic immunostaining method with a positively charged poly(ether sulfone) membrane; the detection limit of GRe was 0.125 μg. The present icELISA is far more sensitive and can allow 50000−500000-fold dilutions of the samples to completely eliminate matrix interference and thus increase the assay accuracy. The GRe content varied in different growing years of *P. ginseng* hairy root samples and ranged from 4.99 to 15.04 mg/g (Table 3). The GRe content in *P. ginseng* hairy root samples generally increases as the growth age increases within the five year life, with which our results agree. In general, the GRe content measured by the icELISA method is slightly higher than that measured by HPLC, which is mainly due to assay cross-reactivity with GRg1, GRd, GRf, and other undetected ginsenosides. The anti-GRe mAb developed by Morinaga et al.[27] also has a similar cross-reactivity. The correlation coefficient (R^2) between the icELISA and HPLC

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Table 3. ELISA, MCS, and HPLC Analysis of GRe in *P. ginseng* Samples

sample[a]	concn from ELISA (mg/g)	concn from MCS (mg/g)	concn from HPLC (mg/g)
one-year-old cultivar	4.99 ± 0.24[b]	5.33 ± 0.41[b]	3.55 ± 0.15
two-year-old cultivar	7.01 ± 0.27	7.32 ± 0.56	4.82 ± 0.27
three-year-old cultivar	10.46 ± 0.67	9.52 ± 0.94	6.61 ± 0.16
four-year-old cultivar	12.33 ± 0.81	12.10 ± 1.01	7.83 ± 0.71
five-year-old cultivar	15.04 ± 0.64	14.88 ± 1.25	10.03 ± 0.66

[a]Each sample was analyzed in triplicate. [b]The data represent the mean ± SD.

results was 0.99. The icELISA is suitable for quality assurance of *P. ginseng* samples.

Activities of Antibody-Functionalized Microcantilevers. ELISA was used to confirm the antibody activities immobilized on the microcantilever. The optical density (OD) values of the thiolated anti-GRe antibody-functionalized microcantilever and unthiolated anti-GRe antibody-functionalized microcantilever were 0.271 and 0.056, respectively. The OD values of the thiolated secondary antibody-functionalized microcantilever and unthiolated secondary antibody-functionalized microcantilever were 0.459 and 0.052, respectively (Figure 2). The results indirectly verified that the thiolated anti-GRe antibody and the thiolated secondary antibody can successfully functionalize the microcantilever and the functionalization method of thiolated secondary antibody can preserve the activity of antibody immobilized on a microcantilever better than that of thiolated primary antibody.

In our previous studies, a protein A-functionalized MCS could accurately measure small molecules at parts per trillion levels.[31] The activity of the antibodies immobilized on the microcantilever via protein A was 1.7 times that via the sulfhydrylation reagent 2-iminothiolane hydrochloride. Protein A has a high affinity for rabbit polyclonal antibodies and has a weak affinity for some subclasses of IgG. Therefore, the application of SPA in MCS is limited. For this reason, we developed an MCS functionalized with thiolated goat antimouse IgG which can specifically bind the Fc region of an antibody without chemical reaction to ensure a good orientation control and avoid the decrease of antibody activity. The method with thiolated goat antimouse IgG was simple and reproducible for functionalization of the antibodies on the Au surface and has common application values for all mouse antibodies in MCS development.

MCS Detection. The sensitivity of the MCS immobilized with thiolated anti-GRe antibody was compared with that of the MCS immobilized with thiolated goat antimouse IgG. Standard samples were added into the fluid cells. Figure 3 shows the deflections of the microcantilever immobilized with thiolated anti-GRe antibody in response to varying concentrations of GRe. Figure 4 shows the deflections of the microcantilever immobilized with thiolated goat antimouse IgG in response to varying concentrations of GRe and the extract of three-year-old *P. ginseng* hairy roots (other samples not shown). The deflection of the MCS immobilized with thiolated anti-GRe antibody was weaker than that of the MCS immobilized with thiolated goat antimouse IgG and then coupled with anti-GRe antibody in the same analyte concentration. When the limit of detection (LOD) is defined as a signal 3 times the background noises, the LOD of the MCS immobilized with thiolated goat antimouse IgG for GRe was approximately 0.5 ng/mL, meaning that MCS is more sensitive than the MCS modified with thiolated anti-GRe antibody.

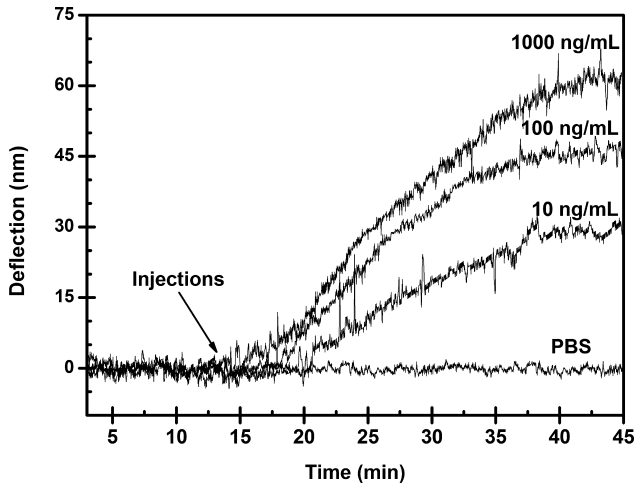

Figure 3. Deflection of the anti-GRe antibody thio-functionalized microcantilever at varying concentrations of GRe in PBS. The standard solutions of 10, 100, and 1000 ng/mL GRe were prepared in PBS for MCS detection.

To verify the advantage of the thiolated secondary antibody as the functionalization reagent, a microcantilever immunosensor for paclitaxel was developed according to the same

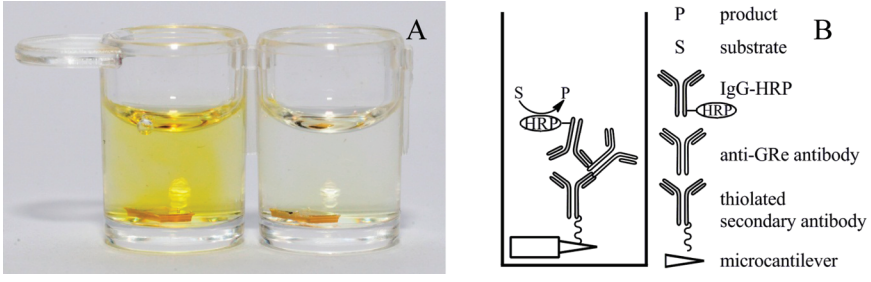

Figure 2. Photographs showing characterization of the anti-GRe antibody functionalized on the microcantilevers via thiolated goat antimouse IgG (A) and schematic of immunological reactions on microcantilevers (B). The activities of the anti-GRe antibody functionalized on the microcantilever via thiolated goat antimouse IgG (vial 1) are compared with those of unthiolated goat antimouse IgG (vial 2). The different colors verified the successful functionalization of the microcantilever. (vial 1 vs vial 2).

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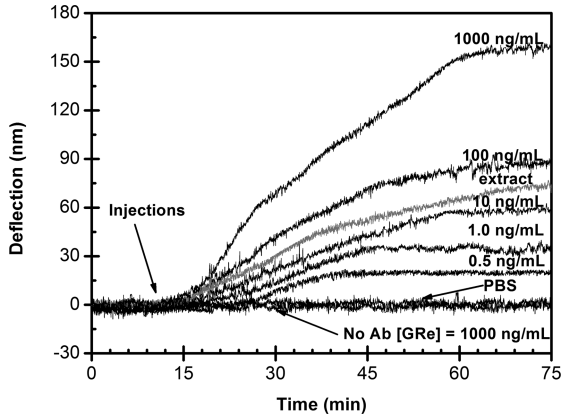

Figure 4. Deflection of the goat antimouse IgG thio-functionalized microcantilever which was then coupled with anti-GRe antibody at varying concentrations of GRe in PBS. The standard solutions of 0.5, 1, 10, 100 and 1000 ng/mL GRe and the extract of three-year-old *P. ginseng* hairy roots were prepared in PBS for MCS detection.

procedure as the above-described GRe microcantilever (Figure 5). The results showed that the MCS immobilized with

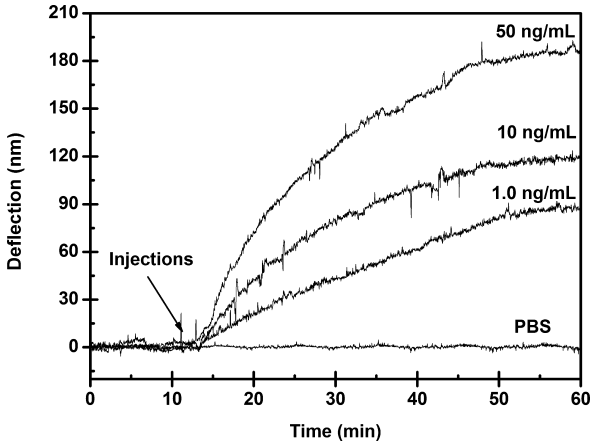

Figure 5. Deflection of the goat antimouse IgG thio-functionalized microcantilever which was then coupled with anti-placlitaxel antibody at varying concentrations of paclitaxel in PBS. The standard solutions of 1.0, 10, and 50 ng/mL paclitaxel were prepared in PBS for MCS detection.

thiolated goat antimouse IgG had a higher sensitivity than our previously studied MCS immobilized with thiolated anti-paclitaxel antibody.[46] In the same paclitaxel concentration of 1 ng/mL, the deflections of the MCS immobilized with thiolated anti-paclitaxel antibody and thiolated goat antimouse IgG and then coupled with anti-paclitaxel antibody were 32 and 89 nm, respectively. The thiolated secondary antibody, which can control the proper antibody orientation and avoid the decrease of antibody activity, is more suitable for the microcantilever immobilization than thiolation of the primary antibody. Therefore, the MCS modified with thiolated goat antimouse IgG was chosen for further study. Table 3 shows that the GRe content varied in different growing years of *P. ginseng* hairy root samples measured by ELISA, MCS and HPLC analysis. The GRe content measured by MCS agreed well with that measured by ELISA. The correlation coefficient (R^2) between the icELISA and MCS results was 0.99. The MCS can be used to accurately monitor the quality of *P. ginseng* samples.

Regeneration and Reproducibility of the MCS. A high degree of uniformity of physical features is required for the microcantilevers. In the present study, the microcantilevers were not regenerated for reuse. Five microcantilevers were used to estimate the degree of uniformity in the same functionalized method and assay conditions. The results showed that, at 10 ng/mL GRe, the bending amplitude of the microbeams was 58 nm with a relative standard deviation (RSD) of 3.5%, indicating acceptable reproducibility.

CONCLUSIONS

To our knowledge, this is the first paper on thiolated goat antimouse IgG-based microcantilevers. The method with thiolated goat antimouse IgG was simple and reproducible. The microcantilever functionalized with thiolated secondary antibody showed better sensitivity than that functionalized with thiolated anti-GRe antibody. The advantage of the secondary antibody thio-functionalized MCS was verified with anti-paclitaxel mAb. The detection range of the MCS functionalized with thiolated secondary antibody was approximately 0.5−1000 ng/mL GRe, which was better than the corresponding icELISA of 0.15−16.1 ng/mL. Analyses of the extracts suggest that the result by the MCS agreed well with that by the icELISA. The results suggest that the thiolated secondary antibody is suitable to functionalize microcantilevers and has common application values for all mouse antibodies in MCS development. The sensitivity, however, would mainly depend upon the microcantilever dimension and shape and the affinity and specificity of the antibodies.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Kiefer, D.; Pantuso, T. *Am. Fam. Physician* **2003**, *68*, 1539−1542.
(2) Wang, C. Z.; Zhang, B.; Song, W. X.; Wang, A.; Ni, M.; Luo, X.; Aung, H. H.; Xie, J. T.; Tong, R.; He, T. C.; Yuan, C. S. *J. Agric. Food Chem.* **2006**, *54*, 9936−9942.
(3) Buettner, C.; Yeh, G. Y.; Phillips, R. S.; Mittleman, M. A.; Kaptchuk, T. J. *Ann. Pharmacother.* **2006**, *40*, 83−95.
(4) Helms, S. *Altern. Med. Rev.* **2004**, *9*, 259−274.
(5) Qu, C. L.; Bai, Y. P.; Jin, X. Q.; Wang, Y. T.; Zhang, K.; You, J. Y.; Zhang, H. Q. *Food Chem.* **2009**, *115*, 340−346.
(6) Morinaga, O.; Uto, T.; Yuan, C. S.; Tanaka, H.; Shoyama, Y. *Fitoterapia* **2010**, *81*, 284−288.
(7) Xie, J. T.; Shao, Z. H.; Vanden, H. T.; Chang, W. T.; Li, J.; Mehendale, S.; Wang, C. Z.; Hsu, C. W.; Becker, L. B.; Yin, J. J.; Yuan, C. S. *Eur. J. Pharmacol.* **2006**, *532*, 201−207.

(8) Joo, K. M.; Lee, J. H.; Jeon, H. Y.; Park, C. W.; Hong, D. K.; Jeong, H. J.; Lee, S. J.; Lee, S. Y.; Lim, K. M. *J. Pharm. Biomed. Anal.* **2010**, *51*, 278−283.
(9) Xie, J. T.; Mehendale, S. R.; Li, X.; Quigg, R.; Wang, X.; Wang, C. Z.; Wu, J. A.; Aung, H. H.; A, R. P.; Bell, G. I.; Yuan, C. S. *Biochim. Biophys. Acta* **2005**, *1740*, 319−325.
(10) http://www.ynet.com/view.jsp?oid=49767670&pageno=1 (accessed March 25th, 2009) and http://bkb.ynet.com/article.jsp?oid=69864540 (accessed October 11th, 2010).
(11) Odani, T.; Tanizawa, H.; Takino, Y. *Chem. Pharm. Bull.* **1983**, *31*, 292−298.
(12) Akao, T.; Kida, H.; Kanaoka, M.; Hattori, M.; Kobashi, K. *J. Pharm. Pharmacol.* **1998**, *50*, 1155−1160.
(13) Li, W.; Fitzloff, J. F. *J. Pharm. Pharmacol.* **2001**, *53*, 1637−1643.
(14) Lau, A. J.; Woo, S. O.; Koh, H. L. *J. Chromatogr., A* **2003**, *1011*, 77−87.
(15) Li, L.; Sheng, Y.; Zhang, J.; Wang, C.; Guo, D. A. *Biomed. Chromatogr.* **2004**, *18*, 849−856.
(16) Li, L.; Zhang, J. L.; Sheng, Y. X.; Guo, D. A.; Wang, Q.; Guo, H. Z. *J. Pharm. Biomed. Anal.* **2005**, *38*, 45−51.
(17) Fuzzati, N. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2004**, *812*, 119−133.
(18) Miao, X. S.; Metcalfe, C. D.; Hao, C.; March, R. E. *J. Mass Spectrom.* **2002**, *37*, 495−506.
(19) Sun, J.; Wang, G.; Haitang, X.; Hao, L.; Guoyu, P.; Tucker, I. *J. Pharm. Biomed. Anal.* **2005**, *38*, 126−132.
(20) Ng, K. M.; Che, C. M.; Wo, S. K.; Tam, P. K.; Lau, A. S. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1545−1550.
(21) Ji, H. Y.; Lee, H. W.; Kim, Y. H.; Jeong, D. W.; Lee, H. S. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2005**, *826*, 214−219.
(22) Lai, C. M.; Li, S. P.; Yu, H.; Wan, J. B.; Kan, K. W.; Wang, Y. T. *J. Pharm. Biomed. Anal.* **2006**, *40*, 669−678.
(23) Li, K.; Chen, X. Y.; Xu, J. H.; Li, X.; Zhong, D. F. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 813−817.
(24) Lee, P. S.; Song, T. W.; Sung, J. H.; Moon, D. C.; Song, S.; Chung, Y. B. *Planta Med.* **2006**, *72*, 204−210.
(25) Jung, D. W.; Lee, J. M.; Sung, C. K. *Anal. Chim. Acta* **2002**, *462*, 157−163.
(26) Fukuda, N.; Tanaka, H.; Shoyama, Y. *Cytotechnology* **2000**, *34*, 197−204.
(27) Morinaga, O.; Tanaka, H.; Shoyama, Y. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2006**, *830*, 100−104.
(28) He, S. P.; Tan, G. Y.; Li, G.; Tan, W. M.; Nan, T. G.; Wang, B. M.; Li, Z. H.; Li, Q. X. *Anal. Bioanal. Chem.* **2009**, *393*, 1297−1303.
(29) Zhao, H. W.; Xue, C. G.; Nan, T. G.; Tan, G. Y.; Li, Z. H.; Li, Q. X.; Zhang, Q. C.; Wang, B. M. *Anal. Chim. Acta* **2010**, *676*, 81−86.
(30) Buchapudi, K. R.; Huang, X.; Yang, X.; Ji, H. F.; Thundat, T. *Analyst* **2011**, *136*, 1539−1556.
(31) Tan, W. M.; Huang, Y.; Nan, T. G.; Xue, C. G.; Li, Z. H.; Zhang, Q. C.; Wang, B. M. *Anal. Chem.* **2010**, *82*, 615−620.
(32) Won, K.; Park, M. J.; Yoon, H. H.; Kim, J. H. *Ultramicroscopy* **2008**, *108*, 1342−1347.
(33) Lee, S.; Cho, J.; Lee, Y.; Jeon, S.; Cha, H. J.; Moon, W. *J. Sens.* **2012**, DOI: 10.1155/2012/217958.
(34) Raiteri, R.; Grattarola, M.; Butt, H. J.; Skládal, P. *Sens. Actuators, B* **2001**, *79*, 115−126.
(35) Velanki, S.; Ji, H. F. *Meas. Sci. Technol.* **2006**, *17*, 2964−2968.
(36) Huang, Y.; Liu, H.; Li, K.; Chen, Y.; Zhang, Q. C.; Wu, X. P. *Sens. Actuators, A* **2008**, *148*, 329−334.
(37) Raiteri, R.; Nelles, G.; Butt, H. J.; Knoll, W.; Skládal, P. *Sens. Actuators, B* **1999**, *61*, 213−217.
(38) Alvarez, M.; Calle, A.; Tamayo, J.; Lechuga, L. M.; Abad, A.; Montoya, A. *Biosens. Bioelectron.* **2003**, *18*, 649−653.
(39) Velanki, S.; Kelly, S.; Thundat, T.; Blake, D. A.; Ji, H. F. *Ultramicroscopy* **2007**, *107*, 1123−1128.
(40) Wu, G.; Datar, R. H.; Hansen, K. M.; Thundat, T.; Cote, R. J.; Majumdar, A. *Nat. Biotechnol.* **2001**, *19*, 856−860.
(41) Weeks, B. L.; Camarero, J.; Noy, A.; Miller, A. E.; De Yoreo, J. J.; Stanker, L. *Scanning* **2003**, *25*, 297−299.
(42) Kawakami, M.; Byrne, K.; Khatri, B. S.; Mcleish, T. C.; Radford, S. E.; Smith, D. A. *Langmuir* **2005**, *21*, 4765−4772.
(43) Hwang, K. S.; Lee, S. M.; Eom, K.; Lee, J. H.; Lee, Y. S.; Park, J. H.; Yoon, D. S.; Kim, T. S. *Biosens. Bioelectron.* **2007**, *23*, 459−465.
(44) Lee, J. H.; Hwang, K. S.; Park, J.; Yoon, K. H.; Yoon, D. S.; Kim, T. S. *Biosens. Bioelectron.* **2005**, *20*, 2157−2162.
(45) Lee, J. M.; Park, H. K.; Jung, Y.; Kim, J. K.; Jung, S. O.; Chung, B. H. *Anal. Chem.* **2007**, *79*, 2680−2687.
(46) Xue, C. G.; Zhao, H. W.; Chen, Y.; Wang, B. M.; Zhang, Q. C.; Wu, X. P. *Sens. Actuators, B: Chem.* **2011**, *156*, 863−866.
(47) Zhao, J.; Li, G.; Wang, B. M.; Liu, W.; Nan, T. G.; Zhai, Z. X.; Li, Z. H.; Li, Q. X. *Anal. Bioanal. Chem.* **2006**, *386*, 1735−1740.
(48) Erlanger, B. F.; Beiser, S. M. *Proc. Natl Acad. Sci. U.S.A.* **1964**, *52*, 68−74.
(49) Beatty, J. D.; Beatty, B. G.; Vlahos, W. G. *J. Immunol. Methods* **1987**, *100*, 173−179.