A Reusable and Specific Protein A-Coated Piezoelectric Biosensor for Flow Injection Immunoassay

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A hydrophilic matrix of periodate-oxidized dextran was used as a double-sided linker to covalently immobilize *Staphylococcus aureus* protein A (SpA) molecules onto a poly-L-lysine-modified piezoelectric crystal surface to improve their stability, activity, and binding specificity with human immunoglobulin G (IgG) in flow injection assays. The prepared sensing crystals displayed best sensitivity and reusability at a flow rate of 140 μL/min. A human IgG concentration as low as 0.3 nM can be detected by this system. Up to 19 successive assay repetitions were achieved without significant loss of sensitivity using the same crystal. The analysis of adsorption kinetics indicates that such a preparation can greatly increase the amount of available active human IgG binding sites on immobilized SpA. Hardly any response arising from unspecific binding was detected. In addition, the sensing crystal prepared by this method was found to retain activity better than one prepared via direct deposition when stored in either wet or dry states. Finally, the prepared SpA-coated crystals were applied to the affinity immobilization of polyclonal goat anti-*Schistosoma japonicum* glutathione-S-transferase (GST) and were able to subsequently detect GST and its genetically engineered mutant either in a purified form or in the crude cell lysate.

Introduction

Over the past decade, immunosensors that utilize immobilized antibodies for the detection of antigens or vice versa have become increasingly practical and useful tools in biotechnology, clinical diagnostics (*1*−*3*), environmental monitoring (*4*, *5*), and the food industry (*6*, *7*). Of the various kinds of detection systems, a piezoelectric (PZ) system, which detects minute amounts of analytes via a decrease in the resonant frequency of PZ oscillating crystals resulting from the adsorption and mass accumulation of analytes on the crystal surface, offers many outstanding advantages desired for commercial immunoassay. These advantages include direct detection, fast response, real-time output, high analyte sensitivity, experimental simplicity, and cost-effectiveness (*8*). Therefore, future widespread use of PZ immunosensors seems inevitable.

One of the most important points in the development of PZ immunosensors is the proper choice of immobilization methods for biomolecules so that they will retain correct orientation, activity, stability, high selectivity, and specificity on the crystal surfaces (*9*, *10*). Because the detected resonant frequencies of PZ crystals decrease with respect to any mass accumulation on the surface, background signals arising from unspecific adsorption or cross-reaction should be eliminated. In addition, when the device is used in combination with a flow injection assay (FIA) technique, which offers the additional advantages of excellent analytical precision and automatic operation, a durable and active sensing layer that can withstand continuous flow, regeneration, and repeated assays is required *(9*, *11*).

It is well-known that *Staphylococcus aureus* protein A (SpA) specifically binds to the F_c regions of antibodies (*12*). Therefore SpA is a universal tool to properly orient antibody molecules so that their antigen binding sites, the F_{ab} regions, are functionally susceptible. Fabrication of PZ crystals with SpA molecules can not only maximize the binding capacity of immobilized antibodies toward antigens but also extend the flexibility and reusability of those prepared crystals, because a whole range of antibodies with different specificities can be bound to and dissociated from the surfaces simply by lowering the pH of the solutions (*11*). Either direct deposition or indirect immobilization via polyethyleneimine adhesion has been used to prepare SpA-coated PZ biosensors for immunoglobulin G (IgG) detection (*13*−*16*). However, only limited levels of SpA activity and very few repetitive uses of the crystals were achieved in those studies. This was probably due to the denaturation of the immobilized SpA molecules, resulting from their direct interaction with hydrophobic or unfavorable solid surfaces.

Indirect immobilization of biomolecules onto the solid surface of sensing devices via a highly hydrophilic hydrogel matrix, such as agarose or dextran, has significantly enhanced the activity, binding capacity, and stability of the prepared sensing layers and reduced interference from unspecific bindings (*17*−*20*). For instance, small haptene molecules, such as short peptides, which were immobilized on plate surfaces using this technique, were able to be functionally detected by their corresponding antibodies in enzyme-linked immunosorbent assays (*18*). In addition, more than 50 cycles of multiple assays for each sensing chip prepared by a similar technique were achieved in a surface plasmon

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resonance sensor (*17*). For PZ biosensors, immobilization of small haptene molecules via hydrogel matrices has also been reported (*21, 22*).

In this paper, we report the utilization of dextran to prepare a hydrophilic SpA-coated PZ biosensor. SpA molecules were indirectly immobilized onto the poly-L-lysine (PLL)-modified electrode surface of PZ crystals by using a hydrogel matrix of periodate-oxidized dextran (POD) as a linker (*23*). Then, human IgG or polyclonal goat antibody against *Schistosoma japonicum* glutathione-S-transferase (GST) was continuously injected and affinity-captured by SpA. A flow-through cell system similar to FIA systems was set up to continuously and repeatedly monitor the specific binding between immobilized SpA and human IgG and to perform the immunoassay of GST. The capacity, reusability, sensitivity, specificity, and storage stability of the resulting SpA-coated PZ crystals prepared via POD were studied and compared with those of the crystals directly deposited with SpA only.

Materials and Methods

Materials. PZ quartz crystals (AT cut, quartz plate diameter 8.5 mm, silver electrode diameter 5.5 mm, 9 MHz resonant frequency) were purchased from Tai-Tein Electric Co. (Taipei, Taiwan). PLL, dextran (molecular weight ≈ 70,000), human serum minus IgG, and human serum minus IgA, IgG, and IgM were from Sigma Chemical Co. (St. Louis, MO). Sodium periodate was from Ferak Laborat GMBH (Berlin, Germany). Glycine was from Amresco (Solon, OH). SpA and human IgG were from Zymed Laboratories Inc. (San Franscisco, CA). *Escherichia coli* BL21, gluthathione Sepharose 4B gels, plasmid pGEX-5X-2 carrying the gene of GST, and polyclonal goat antibody against GST were purchased from Pharmacia Biotech Inc. (Uppsala, Sweden). *E. coli* BL21(DE3) was purchased from NovaGen Inc. (Madison, WI). The plasmid pGSTH carrying the gene of GST/His, a GST mutant fused with a consecutive six-histidine tag at the C-terminus by genetic engineering, was constructed in this laboratory (*24*). Recombinant GST and GST/His were produced from *E. coli* BL21 carrying pGEX-5X-2 and *E. coli* BL21(DE3) carrying pGSTH, respectively. Both proteins were affinity-purified using glutathione Sepharose 4B gels as described elsewhere (*24*).

Preparation of POD. POD was prepared by following the method described in the literature (*23*). Dextran (100 mg) was dissolved in 50 mL of deionized water, followed by the addition of 398 mg of sodium periodate. After 2 h of stirring, the POD solution was dialyzed against deionized water with 12 000−14 000 MWCO dialysis tubes. To modify the surface of the crystals, diluted POD solution prepared by adding 50 mg of dialyzed solution into 100 mL of phosphate buffer (0.1 M, pH 7.2; PB) was used.

Preparation of SpA-Coated PZ Biosensors. Prior to modification, silver PZ crystals were first deposited with a layer of gold by electrochemical plating, then cleaned by immersing in 1.2 M NaOH for 20 min, rinsing with deionized water, and immersing in 1.2 M HCl for 5 min, washed again with deionized water for 10 min (*14*), and finally air-dried.

Two different methods were employed to immobilize SpA molecules onto the gold surface of the crystals. In the first method (*23*), the gold surface was first modified with PLL (0.01%, in 0.1 M carbonate buffer, pH 9.6) at room temperature for 3 h. After being washed with deionized water for 5 min, the crystals were further modified with POD at room temperature for 3 h and washed with deionized water. Then, SpA was covalently attached to the POD-modified crystals by immersing the crystals in SpA solution (0.01% in PB) at 4 °C overnight. In the second method, SpA was passively adsorbed onto the gold surfaces of the crystals simply by immersing the crystals in an SpA solution (0.01% in 0.1 M phosphate buffer, pH near 5.5) as described above. After being rinsed with the same phosphate buffers and then deionized water, the SpA-coated PZ crystals were placed inside the flow cell of the detection system for flow injection assays.

Measuring Setup and Procedures. The experimental setup is schematically depicted in Figure 1a. An SpA-coated PZ crystal was placed inside a thin layer flow cell QCH-01 (internal volume 34 μL, diameter 5 mm, Smart Biotech. Co., Taipei, Taiwan) between two soft rubber O-rings (Figure 1b). The output tube from the cell was connected to a peristaltic pump to control flow rates. The electrodes from the crystal were connected to a QCM analyzer SB01B (Smart Biotech. Co.) via an extended oscillating unit SB01-5. The analyzer consisted of an oscillating circuit and a frequency counter and was controlled by a PC/Pentium 100 computer via a standard RS-232 interface. A program also from Smart, which collected data at intervals of 1 s and at 1 Hz resolution, was used for real-time measurements and data storage.

All measurements were performed at room temperature without any thermostats. Prior to liquid assays, a fresh SpA-coated crystal was placed inside the flow cell. It was first equilibrated with PB and then incubated with glycine solution (0.1 M in 5 mM PB, pH 7.2) at a flow rate of 70 μL/min to block any unreacted aldehyde groups on POD. After equilibration, the flow cell was rinsed with PB again and continuously injected with a solution of human IgG (0.3−333 nM in PB) at a fixed flow rate until a steady resonant frequency was obtained. An excess human IgG solution was continuously passed over the sensing crystal for at least 5 min to ensure that the adsorption equilibrium was obtained. To regenerate the SpA-coated crystals for successive measurements of human IgG solutions, the crystals were washed with 0.1 M glycine−HCl buffer (pH 2.3) to dissociate the SpA−human IgG complex until a steady resonant frequency was obtained and then reequilibrated with PB. To detect *S. japonicum* GST and GST/His, an SpA-coated crystal prepared via POD was first blocked with glycine, equilibrated with PB, and affinity-coated with anti-GST antibodies by allowing a 67 nM (10 μg/mL in PB) antibody solution to flow over the crystal. Then, 2 μg/mL GST or GST/His was passed over that crystal at a flow rate of 140 μL/min.

Analysis of GST Production. To monitor the production of GST from recombinant *E. coli* BL21 following the induction of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 15 mL of the induced cell culture was taken at various time intervals, centrifuged, suspended in 0.75 mL of PB, disrupted by sonication, and recentrifuged. The clear cell lysates were either loaded on a 0.75-mm-thick 12.5% reducing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gel or injected into a PZ biosensor equipped with an SpA-coated crystal that had been further coated with 5 μg/mL anti-GST antibodies as described above. To determine the percentage of GST in the intracellular protein fraction, the SDS−PAGE gel stained with Coomassie brilliant blue R-250 was analyzed using a Kodak Digital Science 1D program from Eastman Kodak Co. (New Haven, CT). Protein concen-

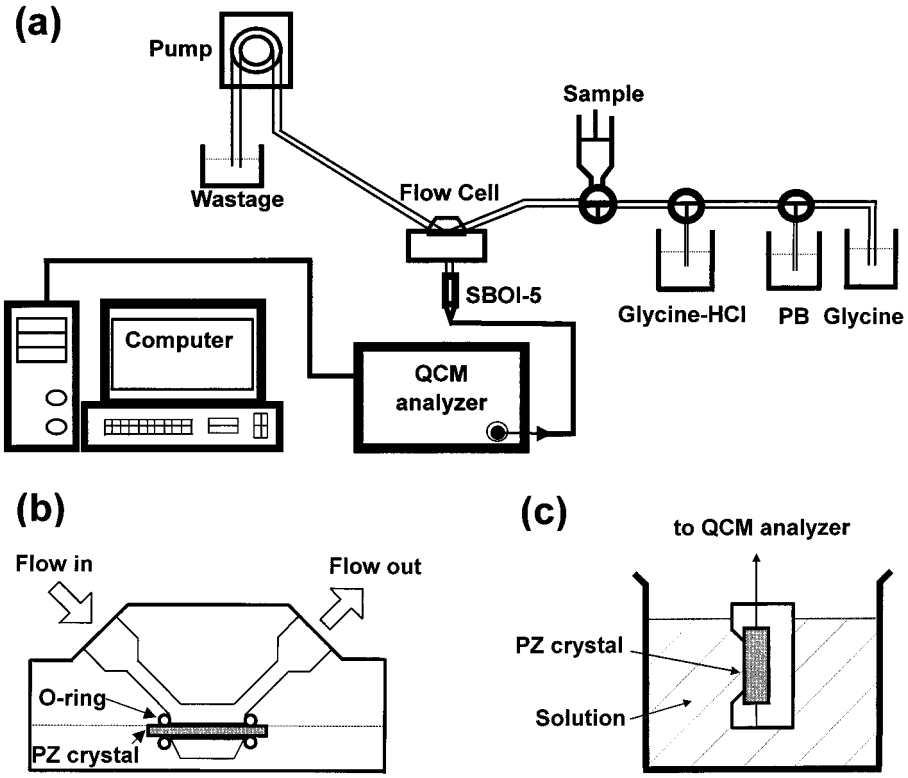


Figure 1. Apparatuses: (a) schematic diagram of flow injection analysis system, (b) cut-away view of flow-through detector cell, and (c) nonflow liquid detector cell.

tration was determined by the method of Bradford (*25*) with bovine serum albumin used as the standard.

Results

Preparation of SpA-Coated Biosensors. At first, the optimal conditions for the preparation of the SpA-coated PZ crystals via POD were studied. The dependence of the changes of resonant frequencies (ΔF, Hz) on the mass accumulated on the crystal surface (ΔM, g) allows one to absolutely determine the deposited materials by using the Sauerbrey equation (*26*):

$$\Delta F = -2.3 \times 10^{-6} F^2 \Delta M/A \qquad (1)$$

where F is the basic resonant frequency of the crystal (Hz) and A is the deposited surface area (cm^2). Therefore, the PZ crystals used here had a theoretic sensitivity of 784 Hz/μg.

To monitor the frequency response in real time and hence to determine the incubation time required at each modification step, a nonflow liquid cell (Figure 1c) was used instead of the flow cell. Inside the cell, a crystal was fixed between two plastic blocks with glue, allowing only one side of the crystal to be in contact with solution while the other side was always dry, and the whole cell was immersed in a modifying solution. The resonant frequency of the crystal decreased to a steady value after 2.2 h incubation during the PLL modification step and reached another steady value after 2.5 h during the subsequent POD modification step. At the final SpA coating step, it took about 12 h for the resonant frequency to become steady. Therefore, in this study, the SpA-coated crystals were prepared by immersing the whole gold crystal (two sides) first in the PLL solution for at least 3 h, then in the POD solution for at least 3 h, and finally in the SpA solution overnight. The results of modifications in each step are summarized in Table 1 (method I). The data show that PLL was successfully immobilized onto the gold surface of the PZ crystals presumably by ionic interactions between positively charged PLL and the locally negatively charged gold surface. Approximately 3.1 pmol of SpA (molecular weight 42 kDa) was immobilized onto each side of the crystal by this preparation method. On the other hand, if SpA molecules were directly immobilized onto the crystal surface by passive adsorption (method II), a $-\Delta F$ value was obtained with a mean 18% less but standard deviation 1. 6-fold higher than those of the value in the above preparation. This suggests that pretreatment of the gold surface of PZ crystals with POD not only can more or less increase the immobilization of SpA onto the crystals but also can reduce the effects of surface variation on immobilization. The increase in the amount of immobilized SpA was insignificant compared to the results in an earlier study (*17*) where a 10- to 20-fold increase was obtained by using a carboxylated dextran matrix. This difference may result from the lack of an electrostatic attraction−concentration effect in the POD matrix to increase the immobilization of SpA. The mean value of the $-\Delta F$ value obtained by direct deposition in this study was comparable with the results presented in earlier works (*14*, *15*) when the difference of basic resonant frequencies is considered.

Table 1. Fabrication of Gold Electrodes of PZ Crystals with SpA

method	step	frequency shift[b] ($-\Delta F$, Hz)	deposited mass[c] (ΔM, μg)
I[a]	PLL	223 ± 19	0.28
	POD	408 ± 28	0.52
	SpA	205 ± 25	0.26
II[a]	SpA	170 ± 40	0.22

[a] Method I: prepared via POD; method II: prepared via direct deposition. [b] Values reported as an average of 17 crystals ± standard deviation. [c] Estimated by using eq 1.

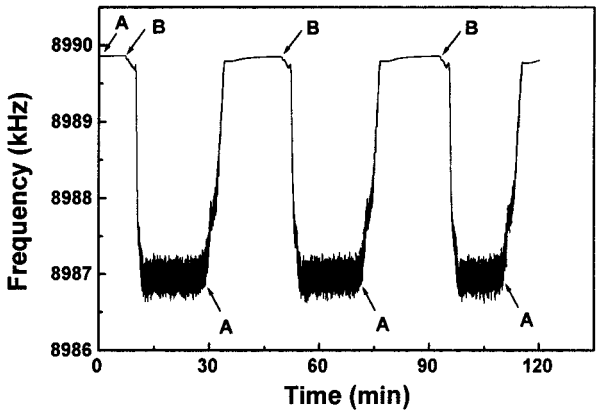


Figure 2. Frequency response of an SpA-coated crystal in repetitive successive rinsing with (A) PB and (B) glycine−HCl buffer.

Flow Injection Assay of Human IgG. Prior to assays using the FIA system, the influence of buffers on the resonant frequency of SpA-coated crystals was studied. Figure 2 shows the resonant frequency of an SpA-coated crystal prepared via POD in response to repetitive successive rinsings with PB and glycine−HCl buffers. The crystal displayed a much lower and more fluctuant resonant frequency in glycine−HCl buffer than in PB. No clear deviation of the frequency was observed when the flowing solution was changed from PB to glycine−HCl buffer and switched back, suggesting that the frequency of the system was stable enough that repetitive assays can be performed. A similar response curve was also observed by using an SpA-coated crystal prepared via direct deposition.

Figure 3 presents a typical frequency response of an SpA-coated crystal prepared via POD in a continuous repetitive assay of human IgG at a concentration of 13.3 nM (2 μg/mL), at a flow rate of 140 μL/min. The injection of human IgG caused the resonant frequency to sharply decrease. Then, a final equilibrium resonant frequency shift was obtained shortly after 2−3 min of injection, indicating the fast response of the detecting system. The complete dissociation of the SpA−human IgG complex was successfully achieved with glycine−HCl buffer with evidence of restoration of the frequency. The restored frequency was not higher than the original one, suggesting that the Schiff bases connecting POD, PLL, and SpA were not reverted during the short-term acid regeneration. Similar response curves were also observed using crystals directly deposited with SpA. Up to 19 successive repetitive assays were achieved without significant loss of sensitivity using one single crystal prepared via POD (Figure 4), suggesting that the prepared sensing crystal was highly reusable and stable in the continuous flow assay. On the other hand, when the sensing chip was replaced with a crystal prepared via direct deposition, only seven successive repetitive assays were achieved, with the average of equilibrium frequency shifts 16% less than that of the former sensing chip. These results suggest that the immobilization of SpA via POD hydrogel instead of directly onto the gold surface can significantly postpone the denaturation of SpA in acid regeneration and consequently enhance the reusability of the sensors in continuously repeating assays.

The effects of flow rates on the performance of sensing chips were also studied by performing the continuous repetitive assay experiments at five different fixed flow rates, ranging from 70 to 560 μL/min. Experiments with flow rates less than 70 μL/min cannot be performed here

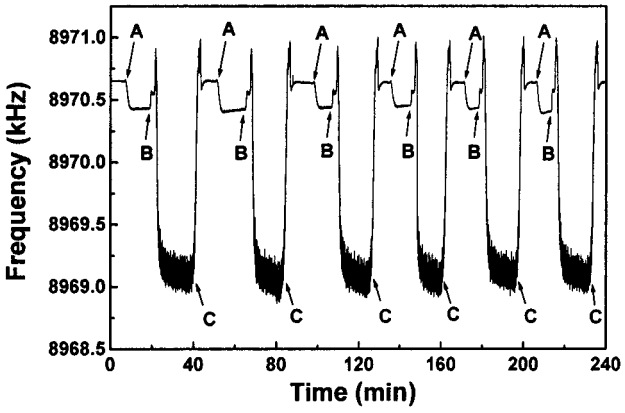


Figure 3. Frequency response of an SpA-coated crystal prepared via POD in continuous repetitive assay of 13.3 nM human IgG at a flow rate of 140 μL/min: (A) injection with human IgG, (B) regeneration with glycine−HCl buffer, and (C) equilibration with PB. Only the first six repeated assay cycles are shown here.

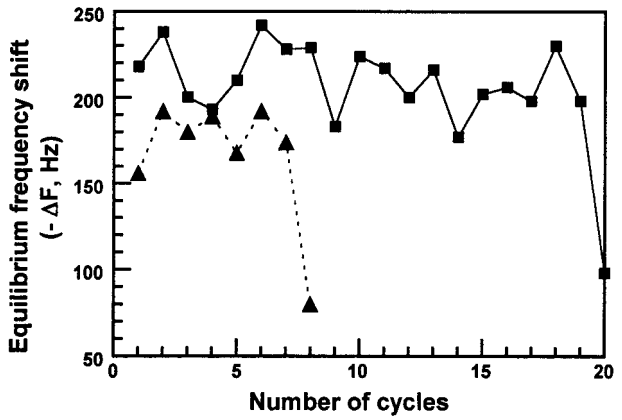


Figure 4. Equilibrium frequency shifts ($-\Delta F$) of SpA-coated crystals prepared via (■) POD modification and (▲) direct deposition in continuous repetitive assay of 13.3 nM human IgG at a flow rate of 140 μL/min. Average of $-\Delta F$ of the first 19 cycles for POD modification is 211 ± 18 Hz (mean ± SD), and that of the first 7 cycles for direct deposition is 178 ± 13 Hz.

because of the limitation of the equipment. Up to 16, 19, and 18 successive repetitive assays were achieved at the flow rates of 70, 140, and 280 μL/min, respectively, whereas only six assays were achieved at 420 μL/min. At a higher flow rate of 560 μL/min, the frequency response of the crystals was small and not reproducible, indicating the instability and low sensitivity of the sensor under such a high flow rate. Figure 5 shows the effects of flow rates on the averages of the equilibrium frequency shifts in those five experiments. At 140 μL/min, the crystals displayed the highest mean value with the smallest standard deviation, suggesting that the sensitivity and the stability of the crystals were optimized under this condition. Therefore, all further assay experiments presented here were performed at a flow rate of 140 μL/min.

Capacity of SpA-Coated Biosensors. The dependence of the response equilibrium frequency shifts on human IgG concentrations is shown in Figure 6 for both SpA-coated biosensors. The lowest concentration analyzed here was 0.3 nM. Linearity was observed in the range of 1.3−46.7 nM for the former sensor and 1.3−20.0 nM for the latter. Over the whole concentration range, the response of the former sensor was greater than that of the latter one, indicating that the sensitivity of the sensor was improved by the presence of the dextran layer. Both plots are rectangular hyperbolics, displaying a

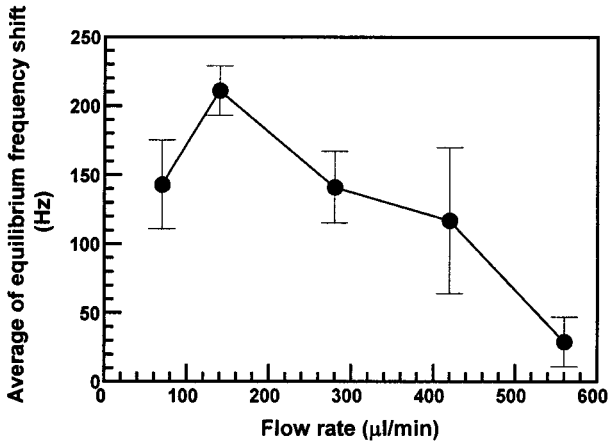


Figure 5. Effect of flow rate on averages of equilibrium frequency shifts in a continuous repetitive assay of 13.3 nM human IgG using SpA-coated crystals prepared via POD. Ranges denote standard deviations.

linear increase at low concentrations and a saturation constant at high concentrations. This suggests that each human IgG binding site on immobilized SpA is independent (*27*). Therefore, the adsorption of human IgG can be illustrated by the use of Langmuir single-site isotherm (*27*)

$$[\text{hIgG}-\text{SpA}] = \frac{K_a[\text{SpA}]_0[\text{hIgG}]}{1 + K_a[\text{hIgG}]} \quad (2)$$

where K_a is the intrinsic adsorption constant for each human IgG binding site (μM^{-1}), [hIgG] is the concentration of human IgG in solution (μM), $[SpA]_0$ is the total surface concentration of active human IgG binding sites on SpA immobilized on the surface (μmol/cm^2), and [hIgG−SpA] is the equilibrium surface concentration of human IgG that is bound to SpA at any human IgG concentration (μmol/cm^2).

Multiplying both sides of eq 2 with the molecular weight of human IgG (150 kDa) and the surface area of the crystal and converting bound human IgG amounts into frequency shifts with the Sauerbrey relationship, eq 2 can be rearranged as

$$-\Delta F = \frac{-\Delta F_{max} K_a[\text{hIgG}]}{1 + K_a[\text{hIgG}]} \quad (3)$$

where $-\Delta F_{max}$ is the saturation frequency shift when all of the active human IgG binding sites on the crystal surface are occupied. Equation 3 can be used directly to fit the curves in Figure 6. The results of fitting are shown in Table 2. Both K_a values are in the order of 10 μM^{-1} (or 10^7 M^{-1}), which is 2 orders lower than the values obtained by the earlier binding study (*28*). Although the amount of SpA immobilized by using POD modification (Sensor I) was only 18% more than the amount obtained by direct deposition (Sensor II), the amount of total active human IgG binding sites ($[SpA]_0$) on Sensor I was 2.7-fold higher than that on Sensor II. The results indicated that the availability or the activity of human IgG binding sites of immobilized SpA was higher on Sensor I than on Sensor II. There are two possibilities that contribute to the great binding difference. First, the POD layer may be in an extended matrix structure where most of the human IgG binding sites of SpA are free to bind with human IgG. Second, the hydrophilic environment that dextran provides may reduce the occurrence of surface denaturation of SpA, which is more likely to occur for direct deposition.

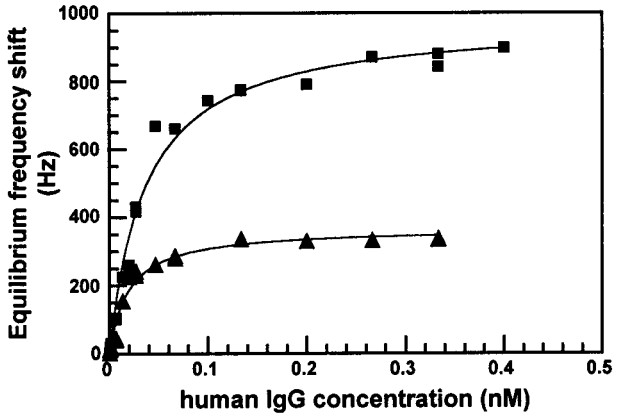


Figure 6. Effect of human IgG concentration on equilibrium frequency shifts of SpA-coated crystals prepared via (■) POD and (▲) direct deposition at a flow rate of 140 μL/min.

Table 2. Kinetic Analysis of Affinity Adsorption between Human IgG and Immobilized SpA

sensor[a]	K_a (μM^{-1})	$-\Delta F_{max}$ (Hz)	$[SpA]_0$ (pmol/cm^2)
I	27.3 ± 2.6	980 ± 25	42.4 ± 0.1
II	53.1 ± 7.4	365 ± 13	15.8 ± 0.6

[a] I: prepared via POD; II: prepared via direct deposition.

Specificity of SpA-Coated Biosensors. The specificity of the prepared biosensors was studied with both human serum minus IgG and human serum minus IgA, IgG, and IgM at two different total protein concentrations. The results of the response equilibrium frequency shifts are summarized in Table 3. The results suggest that in addition to IgG, SpA can also bind with IgA and IgM so that the detected frequency shifts were higher in serum without IgG than in serum without all three immunoglobulins. A previous binding study suggested that the binding affinities of SpA with all three immunoglobulins were similar (*28*). At low serum protein concentrations, the frequency shifts arising from unspecific binding were very insignificant for both sensors. However, at a 10-fold higher concentration, the unspecific frequency shifts detected by the sensor prepared via direct deposition increased significantly and became much larger than those detected by the sensor prepared via POD. The specificity of the sensors prepared via POD was so high that its response to human serum minus IgA, IgG, and IgM was barely detected. Those results suggest that the hydrophilic environment provided by dextran can also eliminate unspecific binding in liquid assays and enhance the sensor specificity.

Storage Stability of SpA-Coated Biosensors. To test the storage stability of the sensing chips, the prepared SpA-coated crystals were stored either in PB or dry at 4 °C for different periods of time and then assayed with 13.3 nM human IgG. Figure 7 shows the decay of the assay activity of the crystals with respect to the storage time. Unfortunately, the activities of all the sensing chips rapidly decreased in the first 2 days. However, under both dry and wet storage conditions, the activities of the crystals prepared via POD decreased at a much slower rate and remained at a 2- to 4-fold higher level than those prepared via direct deposition, suggesting that the hydrophilic dextran layer can indeed help preserve the activity of biomolecules immobilized on the crystal surface. In addition, the results also suggest that

Table 3. Unspecific Binding of SpA-Coated Biosensors[a]

sample	total protein concn (μg/mL)	frequency shift[b] ($-\Delta F$, Hz) sensor I	sensor II
human serum minus IgG	2.3	2	4
	23.0	12	53
human serum minus IgA, IgG, and IgM	2.3	ND[c]	3
	23.0	1	24

[a] The designation of biosensors is the same as in Table 2. [b] Average of three repetitive assays. [c] ND: not detected.

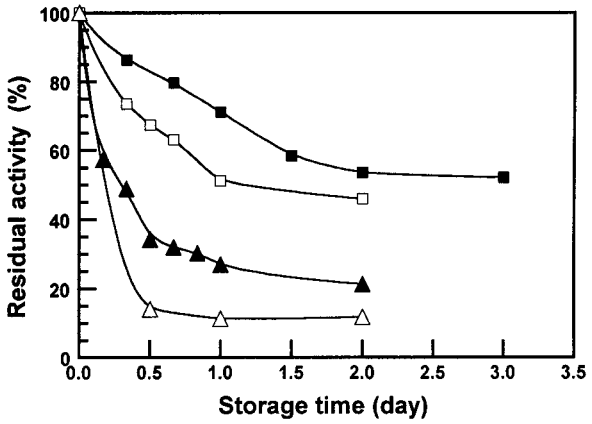


Figure 7. Storage stability of SpA-coated crystals prepared via (■, □) POD and (▲, △) direct deposition at 4 °C. Closed symbols denote storage in PB. Open symbols denote storage in a dry state.

preservation under wet conditions retained the activity of the sensing chips better than under dry conditions.

Immunoassay of GST. An SpA-coated crystal prepared via POD was used to indirectly immobilize polyclonal goat anti-GST antibodies and subsequently to detect GST. The frequency response of the crystal during the immobilization and immunoassay process is shown in Figure 8a. The response curve was highly reproducible, giving an average of frequency shifts at the antibody binding step of 233 ± 23 Hz and at the GST detection step of 83 ± 4 Hz (for four runs). The reinjection of GST following a glycine−HCl regeneration did not lead to any frequency shift, indicating SpA was highly specific to antibodies. Therefore, the unspecific binding was negligible. The shift value arising from the anti-GST antibody binding was less than the one obtained from the human IgG binding at the same concentration (Figure 6), because SpA has a lower binding affinity with goat antibody than with human IgG (*29*). Considering the molecular weight of the recombinant GST as 28 kDa, the molar ratio of the bound GST monomers to the bound anti-GST antibodies is close to 2, which is the number of antigen binding sites on each antibody. This suggests that nearly all the antibodies can be correctly orientated by SpA. In another experiment, we directly immobilized anti-GST antibodies (0.5 mg/mL) onto the POD matrix and obtained a $-\Delta F$ value of 517 Hz, However, no frequency response was detected in the subsequent immunoassay of GST, further confirming the importance of antibody orientation in immunoassay.

In addition, a polyhistidine-tagged GST mutant, GST/His, was also detected with anti-GST antibodies affinity captured by immobilized SpA (Figure 8b). The response curve is similar to that in Figure 8a, with an average of frequency shifts at the GST/His detection step of 82 ± 8 Hz. The result indicates that the existence of a six-

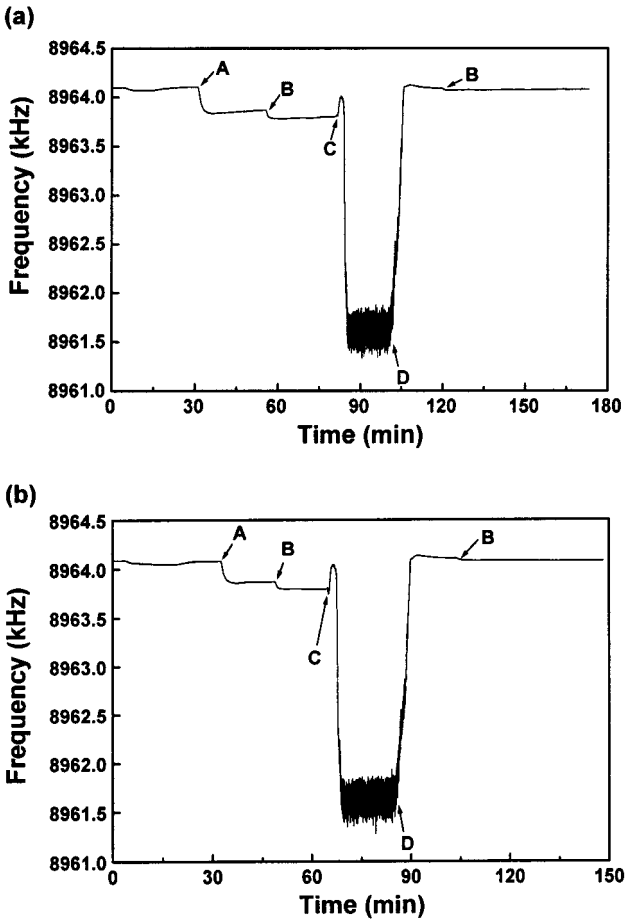
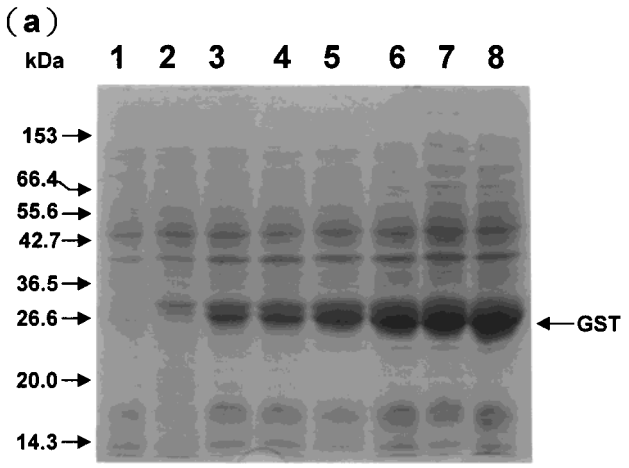


Figure 8. Frequency response of an SpA-coated crystal prepared via POD in affinity immobilization of anti-GST antibody and subsequent immunoassay of 2 μg/mL (a) GST and (b) GST/His at a flow rate of 140 μL/min: (A) injection with anti-GST antibody, (B) injection with (a) GST or (b) GST/His, (C) regeneration with glycine−HCl buffer, and (D) equilibration with PB.

histidine tag at the C-terminus has no significant effect on the binding ability of GST with anti-GST antibodies. These experiments demonstrate the feasibility of direct and rapid immunodetection of recombinant proteins and their mutants using PZ biosensors. In addition, because GST is the major detoxification enzyme and the antigen of *S. japonicum* (*30*), the experiments shown above can be applied to the diagnosis of Schistosomiasis.

Analysis of GST Production. Finally, we studied the possibility of using the prepared PZ biosensor to monitor the production of GST from *E. coli* BL21 carrying pGEX-5X-2. SpA-coated PZ crystals prepared via POD, which had been further coated with anti-GST antibodies, were used to detect GST inside the cells induced by IPTG for different periods of time. For comparison, the production was also monitored by an SDS−PAGE analysis where the total protein concentrations, the percentages of GST in the intracellular protein fraction, and consequently the GST concentrations were determined (Figure 9a). The result of the analysis using the biosensor (Figure 9b) was quite consistent with that of the SDS−PAGE analysis within the first 1 h of induction, both showing an increase in the GST concentration. However, the biosensor analysis failed to continuously show the increase in the GST concentration after 1 h of induction because the antibody-coated crystals were almost saturated with GST, as shown in Figure 10. Therefore, a further diluted sample (dilution ratio > 3000) was required to correctly analyze



Lane #	1	2	3	4	5	6	7	8
Time (hr)	0	0.3	0.6	1.0	1.5	2.0	3.0	4.0
Total protein (mg/ml)	2.1	2.0	3.4	4.8	5.2	6.6	9.3	8.7
GST %	0	8	24	22	30	42	42	41
GST (mg/ml)	0	0.2	0.8	1.1	1.6	2.8	3.9	3.6

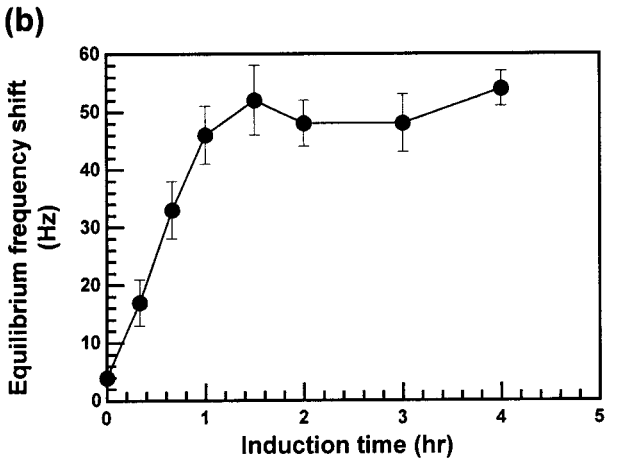


Figure 9. Production of GST from recombinant *E. coli* BL21 cells induced by IPTG for different periods of time. (a) SDS−PAGE of soluble proteins inside the induced cells. A 10 μL portion of clear cell lysate was applied to each lane. (b) Equilibrium frequency shifts of PZ crystals precoated with anti-GST antibodies upon the injections of clear cell lysates of different induction time. All of the clear lysates were 3000-fold diluted by PB before the injection. Ranges denote the standard errors of three experiments.

the GST concentration in the crude lysates of the cells that had been induced for more than 1 h. Nevertheless, the results are sufficient to show that the prepared biosensor can be used to detect a trace amount of GST in the crude cell lysate of *E. coli* without purification and that it is feasible to monitor the GST production using the biosensor if proper dilution is made and the assay time can be greatly reduced.

Conclusions and Discussion

In conclusion, we demonstrated the advantages of hydrogel coatings on the preparation of SpA-coated PZ biosensors. The activity, reusability, sensitivity, specificity, and storage stability of the sensing chips were improved by using the hydrophilic preparation method

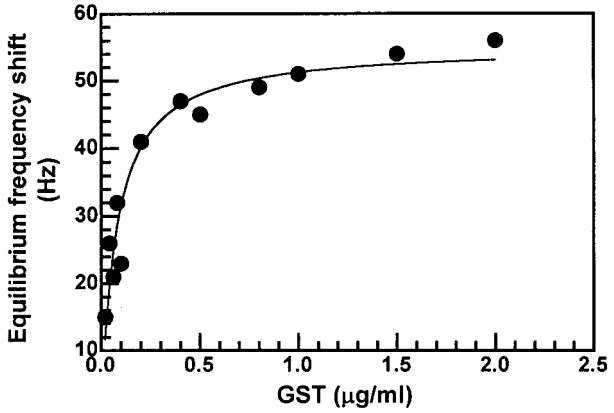


Figure 10. Effect of GST concentration on equilibrium frequency shifts of PZ crystals that had been coated with anti-GST antibodies. A solution of 5 μg/mL anti-GST antibodies was used to flow over SpA-coated PZ crystals prepared via POD for antibody coating.

described here. The technique of hydrogel coatings with POD is very simple and reproducible and can be applied to the immobilization of other proteins on PZ crystals. In addition, the prepared SpA-coated crystals can be further applied to effective immobilization of antibodies, followed by antigen immunoassay, as long as the antibodies can bind with SpA. Both purified antigen and a trace amount of antigen in the crude cell lysate can be detected in this way. The novelty of hydrogel coating was first presented by Löfås and Johnsson (*17*) and has been put in use as the basis of the linking strategy employed in the two main commercial optical biosensors, BIAcore and IAsys. Both sensors used carboxymethyl dextran as the hydrogel linker to covalently immobilize biomolecules onto the transducer surface. The dextran was shown to exhibit a very low nonspecific adsorption of biomolecules and provide a favorable three-dimensional structure for biomolecular interaction. In addition, the negatively charged carboxymethyl group was able to exhibit an electrostatic attraction−concentration effect to greatly enhance the immobilization efficiency (*17*) and provide a functional surface to ease the biomolecule immobilization through a range of activation chemistries (*31*). The POD hydrogel used in this study was not charged, and therefore the amount of SpA immobilized via POD was not much higher than that obtained by direct deposition of SpA onto the gold surface of PZ crystals. However, the greatly improved performances of the POD-modified biosensors in the FIA system, as presented in this study, strongly suggests the necessity of hydrogel coating in the preparation of PZ biosensors and sufficiently shows the feasibility of the use of POD as a hydrogel linker.

Continuous monitoring of bioprocess or biological interaction is one of the important applications for FIA biosensors. The high reusability and the fast response of the sensing chips prepared via POD in the FIA system suggest that an automatic, cost-effective, direct-detection, and continuous PZ biosensing system can be feasibly set up for on-line monitoring. To set up, an automatic sampling system including devices for sample pretreatment, such as cell separation and dilution, is required. In addition, the effect of fluidic viscosity on the resonant frequency of PZ crystals has to be considered, if a nondiluted process sample is directly used for measurement (*32, 33*). For practical applications, an automatic continuous FIA system should be developed to analyze a large number of samples by using the same sensing chip. In this way, the requirement for concentration calibration

and the interruption frequency on measurement as a result of chip changes can be minimized. On the basis of this viewpoint, the PZ biosensing chips presented in this study need to be further improved because of the rapid decrease of the stability of the prepared SpA-coated crystals upon storage and after 20 repetitive uses. It is possible that the prepared hydrogel matrix is not thick enough or that the structure of PLL is too loose to fully cover the gold layer, leading to failure in protecting the protein completely from surface denaturation. Therefore, a material with a more compact structure than PLL, such as long-chain thiols, that can form a self-assembled monolayer onto the gold layer via chemical adsorption (*34*), can be used instead. In the BIAcore sensing chip, whose reusability is more than 50 repetitive cycles, the gold surface is first coated with hydroxyl-terminated alkanethiols for subsequent attachment of carboxymethyl dextran (*17*). We have recently developed another hydrogel-coated PZ biosensor that was prepared with hydrazide-terminated alkanethiols and POD and exhibited fairly good stability upon 30-day storage and after 40 repetitive uses (manuscript in preparation). Finally, if the flow cell used in this study can be further miniaturized so that the optimal flow rate can be reduced to several μL per sec to reduce the consumption of samples and the detection time, the PZ system can be used as an alternative tool to enzyme-linked immunosorbent assays for immunoassay.

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References and Notes

(1) König, B.; Grätzel, M. Development of a piezoelectric immunosensor for the detection of human erythrocytes. *Anal. Chim. Acta* **1993**, *276*, 329−333.

(2) König, B.; Grätzel, M. A novel immunosensor for herpes viruses. *Anal. Chem.* **1994**, *66*, 341−344.

(3) Uda, T.; Hifumi, E.; Kobayashi, T.; Shimizu, K. An approach for an immunoaffinity AIDS sensor using the conservative region of the HIV envelope protein (pg41) and its monoclonal antibody. *Biosens. Bioelectron.* **1995**, *10*, 477−483.

(4) Wittmann, C.; Schmid, R. D. Bioaffinity sensors for enviromental monitoring. In *Handbook of Biosensors and Electronic Noses: Medicine, Food, and the Environment*; Kress-Rogers, E., Ed.; CRC Press: New York, 1996; Chapter 15.

(5) Steegborn, C.; Skládal, P. Construction and characterization of the direct piezoelectric immunosensor for atrazine operating in solution. *Biosens. Bioelectron.* **1997**, *12*, 19−27.

(6) Plomer, M.; Guilbault, G. G.; Hock, B. Development of a piezoelectric immunosensor for the detection of enterobacteria. *Enzyme Microb. Technol.* **1992**, *14*, 230−235.

(7) Minunni, M.; Mascini, M.; Carter, R. M.; Jacobs, M. B.; Lubrano, G. J.; Guilbault, G. G. A quartz crystal microbalance displacement assay for *Listeria monocyrogenes*. *Anal. Chim. Acta* **1996**, *325*, 169−174.

(8) Hlavay, J.; Guilbault, G. G. Application of the piezoelectric crystal detector in analytical chemistry. *Anal. Chem.* **1977**, *49*, 1890−1898.

(9) Luong, J. H. T.; Guilbault, G. G. Analytical applications of piezoelectric crystal biosensors. In *Biosensor Principles and Applications*; Blum, L. J., Coulet, P. R., Eds.; Marcel Dekker: New York, 1991; Chapter 6.

(10) Scouten, W. H.; Luong, J. H. T.; Brown, R. S. Enzyme or protein immobilization techniques for applications in biosensor design. *Trends Biotechnol.* **1995**, *13*, 178−185.

(11) Palmer, D. A.; French, M. T.; Miller, J. N. Use of protein A as an immunological reagent and its application using flow injection, a review. *Analyst* **1994**, *119*, 2769−2776.

(12) Forsgren, A.; Sjöquist, J. "Protein A" from *S. aureus*. I. Pseudo-immune reaction with human γ-globulin. *J. Immunol.* **1966**, *97*, 822−827.

(13) Muramatsu, H.; Dicks, J. M.; Tamiya, E.; Karube, I. Piezoelectric crystal biosensor modified with protein A for determination of immunoglobulins. *Anal. Chem.* **1987**, *59*, 2760−2763.

(14) Davis, K. A.; Leary, T. R. Continuous liquid-phase piezoelectric biosensor for kinetic immunoassays. *Anal. Chem.* **1989**, *61*, 1227−1230.

(15) Prusak-Sochaczewski, E.; Luong, J. H. T. A new approach to the development of a reusable piezoelectric crystal biosensor. *Anal. Lett.* **1990**, *23*, 401−409.

(16) Dubrovsky, T.; Tronin, A.; Dubrovskaya, S.; Vakula, S.; Nicolini, C. Immunological activity of IgG Langmuir films oriented by protein A sublayer. *Sens. Actuators, B* **1995**, *23*, 1−7.

(17) Löfås, S.; Johnsson, B. A novel hydrogel matrix on gold surfaces in surface plasmon resonance sensors for fast and efficient covalent immobilization of ligands. *J. Chem. Soc., Chem. Commun.* **1990**, *21*, 1526−1528.

(18) Gregorius, K.; Mouritsen, S.; Elsner, H. I. Hydrocoating: a new method for coupling biomolecules to solid phases. *J. Immunol. Methods* **1995**, *181*, 65−73.

(19) Hartmann, A.; Bock, D.; Schulze, M.; Tebbe, H.; Wegner, G.; Seeger, S. Cellulose as a new matrix for one-step immobilized antibodies and application in high specific immunosensors. *Transducers '95. Eurosensors IX* **1995**, pp 505−508.

(20) Piehler, J.; Brecht, A.; Geckeler, K. E.; Gauglitz, G. Surface modification for direct immunoprobes. *Biosens. Bioelectron.* **1996**, *11*, 579−590.

(21) Horácek, J.; Skládal, P. Improved direct piezoelectric biosensors operating in liquid solution for the competitive label-free immunoassay of 2,4-dichlorophenoxyacetic acid. *Anal. Chim. Acta* **1997**, *347*, 43−50.

(22) Rickert, J.; Brecht, A.; Göpel, W. Quartz crystal microbalances for quantitative biosensing and characterizing protein multilayers, *Biosens. Bioelectron.* **1997**, *12*, 567−575.

(23) Elsner, H. I.; Mouritsen, S. A method of chemical coupling on solid phases. International Patent Application WO94/03530, 1994.

(24) Chen, H.-M.; Luo, S.-L.; Chen, K.-T.; Lii, C.-K. Affinity purification of *Schistosoma japonicum* glutathione-S-transferase and its site-directed mutants with glutathione affintiy chromatography and immobilized metal affintiy chromatography. *J. Chromatogr., A* **1999**, *852*, 151−159.

(25) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(26) Sauerbrey, G. Z. Use of quartz vibrator for weighing thin films on a microbalance. *Z. Phys.* **1959**, *155*, 206−211.

(27) Palmer, T. *Understanding Enzymes*, 4th ed.; Prentice Hall/Ellis Horwood: Hertfordshir, 1995; Chapter 12.

(28) Ljungberg, U. K.; Jansson, B.; Niss, U.; Nilsson, R.; Sandberg, B. E. B.; Nilsson, B. The interaction between different domains of *Staphylococcal* protein A and human polyclonal IgG, IgA, IgM, and F(ab′)$_2$: separation of affinity from specificity. *Mol. Immunol.* **1993**, *30*, 1279−1285.

(29) Åkerström, B.; Björck, L. A physicochemical study of Protein G, a molecule with unique immunoglobulin G-binding properties. *J. Biol. Chem.* **1986**, *261*, 10240−10247.

(30) Smith, D. B.; Davern, K. M.; Board, P. G.; Tiu, W. U.; Garcia, E. G.; Mitchell, G. F. Mr 26,000 antigen of *Schistosoma japonicum* recognized by resistant WEHI 129/J mice is a parasite glutathione S-transferase. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 8703−8707.

(31) O'Shannessy, D. J.; Brigham-Burke, M.; Peck, K. Immobilization chemistries suitable for use in the BIAcore surface plasmon resonance detector. *Anal. Biochem.* **1992**, *205*, 132−136.

(32) Keijikanazawa, K.; Gordon, J. G. The oscillation frequency of a quartz resonator in contact with a liquid. *Anal. Chim. Acta* **1985**, *175*, 99−105.

(33) Hayward, G. L.; Chu, G. Z. Simultaneous measurement of mass and viscosity using piezoelectric quartz crystals in liquid media. *Anal. Chim. Acta* **1994**, *288*, 179−185.

(34) Bain, C. D.; Troughton, E. B.; Tao, Y.-T.; Evall, J.; Whitesides, G. M.; Nuzzo, R. G. Formation of monolayer films by the spontaneous assembly of organic thiols from solution onto gold. *J. Am. Chem. Soc.* **1989**, *111*, 321−335.

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