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# Immobilization of Thiabendazole-Specific Monoclonal Antibodies to Silicon Substrates via Aqueous Silanization

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## ABSTRACT

Monoclonal antibodies specific for thiabendazole were immobilized to silicon, silicon dioxide, stoichiometric silicon nitride, and silicon-rich silicon nitride surfaces. This work provides the foundation for the development of a homogeneous sensor system for rapid detection and quantification of thiabendazole residues in produce and animal tissue. Immobilization was performed via aqueous silanization of the substrate followed sequentially by treatment with glutaraldehyde and contact with antibody solution in the presence of detergent. Surfaces were challenged with thiabendazole-horseradish peroxidase conjugate in an ELISA format to estimate immobilized antibody load. A stable and reproducible surface loading of $2 \times 10^{11}$ antibodies/cm$^2$ was obtained only after surfaces received postimmobilization treatments to remove nonspecifically adsorbed antibody. No difference in surface loading was noted when using 30% hydrogen peroxide rather than nitric acid for silanol activation. Little difference was noted among the antibody loadings achieved on the various silicon substrates. Bound antigen-enzyme conjugate was eluted with 0.1*N* acetic acid and reproducible surface activity was measured for up to four consecutive antigen challenges. Immobilized antibody surfaces were stabilized with 2% sucrose, dehydrated at 37°C and stored in vacuum or stored at 4°C in phosphate buffered saline containing 0.01% sodium azide without significant loss of activity.

**Index Entries:** Protein immobilization; thiabendazole; silicon dioxide; silicon nitride.

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## INTRODUCTION

Thiabendazole is regularly used as both a postharvest fungicide for many crops and an anthelmintic in cattle for control of a variety of gastrointestinal worms (*1*). Owing to its mechanism of action (primarily inhibition of the formation of microtubules [*2*]) thiabendazole and other benzimidazoles exhibit chronic toxicity effects such as teratogenesis in mice (*3*). Therefore, maximum permissible levels of thiabendazole residues in produce (*4*) and edible tissue have been estabilshed (*5*).

To verify that produce and meat products are below tolerance levels, methods for residue detection have been developed. Analysis is typically performed using high performance liquid chromatography (HPLC) with UV detection (*6*). The time and expertise required for HPLC analysis severely limits the number of samples that can be screened. Methods for rapid residue detection in produce and meat samples would facilitate monitoring efforts and enable an increase in the number of samples screened.

Polyclonal (*7*) and monoclonal antibodies (MAbs) (*8*) specific for thiabendazole and enzyme-linked immunosorbent assays (ELISAs) for detection and quantification of thiabendazole residues in a variety of food matrices have been developed. These assays offer a significant decrease in analysis time and operator expertise requirements. However, the need for a one-step, homogeneous, direct detection sensor system persists. To take advantage of the sensitivity and specificity of the already developed monoclonal antibodies, such a system would utilize the thiabendazole-specific MAbs as the active sensing element.

As a first step in the development of a homogeneous sensor system for thiabendazole residue analysis, a technique for antibody immobilization to a sensor surface must be developed. The goal of this study then, was development of a technique for immobilization of a stable, active, uniform, and reproducible layer of thiabendazole-specific antibody. It was also desirable to develop protocols for storage of antibody coated surfaces and for regeneration of active surfaces after challenge with corresponding antigen. Finally, it was preferred that the immobilization technique minimize handling and disposal of toxic or corrosive chemicals.

## BACKGROUND

Covalent immobilization of proteins to solid substrates via silanization followed by glutaraldehyde treatment was developed over 20 yr ago (*9*). The technique continues to be one of the most prevalent chemistries for protein attachment because of its simplicity and resultant high protein loads. Aqueous silanization with a trifunctional silane (aminopropyltriethoxysilane) was selected since it results in more stable and uniform

immobilized protein layers (*10*) and precludes the use and disposal of organic solvents.

Before treating a surface with a silanating agent, the surface is given an aggressive acid wash, typically 80°C in nitric acid (*10,14*). This acid treatment serves to clean the surface and, more important, to activate the surface silanol groups so that they are available for reaction with the silanating agent. In this study, alternative cleaning/activation treatments were also investigated. Sulfuric acid:hydrogen peroxide (5:1) and 30% hydrogen peroxide were tested. The sulfuric acid:hydrogen peroxide mixture is commonly referred to as piranha and is a vigorous oxidizing solution regularly employed in the semiconductor industry for cleaning silicon substrates (*11*). The effectiveness of piranha in removing organic and heavy metal contaminants from silicon substrates has been well documented (*12*) and it also accomplished silanol activation. The hydrogen peroxide alone demonstrated that a milder and more easily disposed solution performed as effectively as the more hazardous acid solutions.

A persistent problem associated with covalent antibody immobilization techniques is minimizing nonspecifically adsorbed antibody (*13*). By contacting activated surfaces with protein in the presence of detergent, a decrease in the adsorbed antibody load has been reported (*14*). Detergents were also utilized in this study. Additionally, removal of adsorbed antibody with postimmobilization treatments of acetic acid, urea, or heated phosphate buffered saline (curing) (*15*) were investigated; curing was the most effective.

Nonspecific adsorption of secondary proteins is also a concern. For example, nonspecific adsorption of additional proteins from the test sample can interfere with the ability of immobilized antibody to capture analyte and nonspecific binding of antigen–enzyme conjugate results in misinterpreted substrate conversion signals during ELISAs. Therefore, after antibody immobilization, nonreacted aldehyde groups are quenched by reaction with a low molecular weight amine (usually ethanolamine) and adsorption sites are blocked by treatment with excess protein (e.g., bovine serum albumin, BSA). These treatments were also investigated in this study but had little effect.

Numerous investigators have used aqueous silanization to immobilize proteins to glass slides or beads (silicon dioxide) and recently silanization has also been used with stoichiometric silicon nitride substrates (*14*). For sensor systems, silicon nitride is often the preferred material because of its resistance to contaminant ion diffusion (*16*) and ability to withstand microcrack formation (*17*). However, stoichiometric silicon nitride films display high residual stress after formation (*18*) and are not suitable for sensors that utilize thin silicon nitride membranes, such as surface acoustic wave devices (*19*). For these devices, a nonstoichiometric, silicon-rich silicon nitride has been developed (*20*) that exhibits a low residual stress.

This study concluded there was little difference among the antibody loadings achieved on silicon, silicon dioxide, stoichiometric silicon nitride, and silicon-rich silicon nitride. The ability of the immobilization procedure to be used with a variety of substrates was verified so that a variety of sensor strategies could be investigated in the future.

## MATERIALS AND METHODS

### Antibodies

Monoclonal antibodies specific against thiabendazole were produced from hybridoma cells. Hapten synthesis, antibody production, and characterization (*8b*) and antibody purification from ascitic fluid (*21*) have been reported previously.

### Substrates

All tests were performed with 8 × 8 mm chips diced from 100 mm, <100>, *p*-type silicon wafers (15–30 Ω-cm). New wafers were cleaned in 5:1 $H_2SO_4$:$H_2O_2$ for 10 min then in 10:1 $H_2O$:HF until the water surface was hydrophobic (~10 s). Wafers and chips were rinsed with deionized water following all chemical treatments and after removal from storage solutions. Films were prepared immediately after wafer cleaning.

*Silicon Dioxide ($SiO_2$)*

Cleaned wafers were oxidized in a resistively heated tube furnace at 1000°C, 5 min dry $O_2$, 11 min steam, 5 min dry $O_2$ followed by a 20 min *in situ* $N_2$ anneal.

*Silicon (Si)*

Silicon substrates were prepared just prior to immobilization by stripping silicon dioxide chips in 5:1 $NH_4F$:HF until hydrophobic.

*Stoichiometric Silicon Nitride ($Si_3N_4$)*

Silicon nitride films were formed on cleaned wafers in a low pressure chemical vapor deposition (LPCVD) furnace at 800°C, 300 mtorr using $NH_3$ (75 sccm) and dichlorosilane (25 sccm).

*Low-Stress Silicon Nitride (SiN)*

Silicon-rich silicon nitride films were formed on cleaned wafers by LPCVD at 835°C, 140 mtorr using $NH_3$ (25 sccm) and dichlorosilane (100 sccm).

Thickness of all oxide and nitride films was 100–120 nm. Wafers were coated with positive photoresist (820, KTI Chemicals Inc., Sunnyvale, CA), and baked at 120°C for 30 min in a convection oven to protect films during backside etching. Silicon dioxide films were stripped from the

wafer backsides by immersing in 5:1 $NH_4F$:HF until the backside was hydrophobic. Silicon nitride films were stripped from the water backside in a parallel plate plasma reactor (Planaretch IIA, Technics, Dublin, CA) at 100 W for 3 min with $SF_6$ (13 sccm) and He (21 sccm), followed by immersion in 5:1 $NH_4F$:HF until hydrophobic. Waters were mounted on dicing paper, diced into 8 × 8 mm chips with an automated dicing saw (Model DAD-2H/6, Disco Abrasives System Inc., Santa Clara, CA) and stored until needed for immobilization. Once diced, all chips received individual chemical treatments by using 24-well polystyrene tissue culture plates (Costar, Cambridge, MA) as reaction vessels; each chip was placed in a separate well with 1.2 mL of solution and plates were agitated on shaker tables during chemical treatments. Though all chips were silicon substrates with a thin film, they are described as silicon dioxide chips, silicon nitride chips, and so on.

## Immobilization Procedure

### *Surface Activation*

Chips were removed from dicing paper and soaked in acetone for 1 min to strip the protective photoresist. To activate surface silanol groups, three different treatments were compared: nitric acid, 80°C, 1 h; 5:1 $H_2SO_4$:$H_2O_2$, 20 min; and 30% $H_2O_2$, room temperature, 1 h. Thirty percent $H_2O_2$ was used in all other comparisons.

### *Silanization*

Chips were soaked in 10:1 $H_2O$:aminopropyltriethoxysilane (APTS, Sigma, St. Louis, MO) adjusted to pH 7.0 with 10% acetic acid at 80°C for 4 h. Aqueous silanization is usually performed at pH 4.0 (*10*), but recent work suggests that pH 7.0 is preferable (*14*). The higher pH was selected for this study. One set of chips was dehydrated overnight in a convection oven at 110°C after silanization.

### *Glutaraldehyde*

Chips were soaked in 10% glutaraldehyde (Grade II, Sigma) at room temperature for 1 h. Chips were rinsed individually in a small sample vial on a vortexer to insure removal of excess glutaraldehyde and avoid crosslinking of immobilized antibody. The glutaraldehyde step was omitted for some samples to obtain an estimate of noncovalently bound protein.

### *Protein Contact*

Chips were soaked in 0.25 mg/mL antibody in phosphate-buffered saline (PBS, 0.15*M* NaCl, 5 m*M* sodium phosphate, pH 7.0) containing 0.5% Tween 60 (*14*) for 40 min at room temperature, then rinsed with PBS containing 0.05% Tween 20 (PBS-Tween 20) then rinsed with PBS only. Again, chips were rinsed individually in a small sample vial on a vortexer.

*Backside Coating*

Chips were touched to the surface of a pool of molten paraffin, M. P. 53–57°C so that a layer of wax was applied to the chip backside but no wax came in contact with the chip topside.

## Postimmobilization Treatments

For tests related to removal of nonspecifically adsorbed antibody, chips received one of the following treatments: 5 min soak in 0.1*N* acetic acid, 5 min soak in 6*M* urea, 4 h soak in 56°C PBS, overnight soak in PBS-Tween20, 1 h soak in substrate solution, or 1 h soak in TBZ-HRP solution followed by 5 min soak in 0.1*N* acetic acid. (Substrate and TBZ-HRP solutions are defined below.) For tests related to preventing nonspecific adsorption of secondary proteins, chips received one of the following treatments: 1 h soak in 0.2*M* ethanolamine pH adjusted to 9.0 with 12*N* HCl; or 1 h soak in PBS containing 10 mg/mL bovine serum albumin (BSA, Sigma). Chips were treated only prior to the initial testing of the surfaces for antibody activity.

*Storage*

Chips were stored individually in tissue culture plate wells with 1.2 mL PBS containing 0.01% sodium azide (PBS-azide) at 4°C until ready to measure activity. For one set of chips, a dehydration protocol (*8b*) was tested. Chips were soaked in a 2% w/v sucrose solution for 30 min, shaken dry, then dehydrated at 37°C for 1 h. Chips were stored in an evacuated dessicator jar (P ~ 100 mtorr) for 1 wk before testing activity.

## Determination of Immobilized Antibody Activity

Chips were removed from PBS-azide storage buffer and incubated with a solution of horseradish peroxidase (Type VI-A, Sigma) conjugated to thiabendazole (Merck Sharp and Dohme, Rahway, NJ) (TBZ,HRP) for 2 h. The conjugation procedure (*22*) resulted in a TBZ:HRP molar ratio of approx 6:1. TBZ-HRP solution consisted of approx 100 ng/mL TBZ-HRP in PBS containing 10 mg/mL BSA and 0.2% thimerosal (Sigma). One set of chips was incubated with a solution of horseradish peroxidase conjugated to rabbit antimouse IgG (RαMIgG-HRP [Zymed, So. San Francisco, CA], 1:1000 in PBS containing 10 mg/mL BSA). Following incubation in TBZ-HRP (or RαMIgG-HRP), chips were rinsed with PBS-Tween 20, rinsed with PBS, then placed in individual wells containing substrate solution. Substrate solution consisted of 1 m*M* 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid, ABTS (Sigma)) and 6.7 m*M* $H_2O_2$ in 60 m*M* sodium citrate buffer, pH 4.2. Fifty microliter aliquots were then removed from each well at 5-min intervals and placed in 96-well tissue culture plates. After 1 h total reaction time, the absorbance at 414 nm for each well was mea-

sured with an automated microplate reader (Vmax, Molecular Devices, Menlo Park, CA). Activity was defined as rate of change of absorbance per unit area ($\Delta A_{414}$/min·cm$^2$).

*Controls*

Two sets of control chips were prepared to investigate nonspecific adsorption of TBZ-HRP to protein-coated surfaces. During the protein immobilization procedure, in place of the thiabendazole-specific monoclonal antibody, one set of chips was contacted with a monoclonal antibody that does not bind TBZ (specific for soybean trypsin inhibitor [*21*]) and a second set of chips was contacted with PBS containing 10 mg/mL BSA. These chips were tested for activity by incubating with the TBZ-HRP conjugate and monitoring production of colored product after substrate addition as described above. Chips completely coated with paraffin were also prepared and incubated in the TBZ-HRP conjugate; these chips were to estimate nonspecific adsorption of enzyme to the backsides of the test chips.

## Regeneration of Active Surfaces

After challenge with substrate solution, chips were soaked in 0.1*N* acetic acid for 5 min, then stored in PBS-azide at 4°C until ready for the next antigen challenge. Subsequent challenges were performed within 1–3 d of the preceding test.

*Controls*

Before a subsequent antigen challenge was performed, chips were soaked in substrate solution and monitored for production of colored product to verify that hapten–enzyme conjugate had been eluted by the acetic acid treatment.

## Determination of Conjugated TBZ-HRP Activity

TBZ-HRP solution (20 μL) was added to 50–200 μL of substrate solution in microplate wells and rate of change of absorbance ($\Delta A_{414}$/min) was monitored with the microplate reader. Enzyme turnover number was estimated from solution activity and oxidized ABTS extinction coefficient (*23*).

## Ellipsometry Measurements

A set of silicon dioxide chips was measured at 632.8 nm by ellipsometry (Model L116A, Gaertner, Chicago, IL) after each of the chemical treatments of the immobilization procedure. Refractive index was fixed at 1.46 for all measurements. The same chips were then incubated in a solution of TBZ-BSA (10 μg/mL in PBS) for 2 h at room temperature, rinsed with PBS-Tween 20, rinsed with PBS, dried with $N_2$, and measured again by ellipsometry.

## RESULTS AND DISCUSSION

### Controls

Chips with immobilized control MAb (specific for soybean trypsin inhibitor), BSA, or completely coated with paraffin showed no activity when challenged with TBZ-HRP and substrate solution. Chips that were challenged with substrate solution following acetic acid treatment to verify TBZ-HRP elution also displayed zero activity.

The control tests indicate that nonspecific adsorption of TBZ-HRP to antibody coated surfaces or the waxed chip backsides was not a concern. Also encouraging was the lack of any residual activity of chips after an acetic acid wash but prior to a subsequent challenge with hapten-enzyme conjugate. This result indicates that the acetic acid wash successfully eluted the bound antigen from the antibody coated surface. An alternate explanation is that the acetic acid treatment simply inactivated the bound enzyme; however, the ability of the surface to recapture hapten-enzyme (Figs. 1-6) is further evidence that antigen release took place.

### $SiO_2$ Chips: Silanol Activation and Chip Storage

The three silanol activation methods resulted in comparable chip surface activity (Fig. 1, column sets 1-3). The nitric acid or sulfuric acid: hydrogen peroxide may be justified if the surface needs an aggressive cleaning. However, if a semiconductor-quality substrate is employed, the substrate was cleaned prior to film formation, and the substrate has just been removed from a high temperature oxidation or nitride deposition furnace additional cleaning is not necessary. If silanol activation rather than cleaning is all that is desired, hydrogen peroxide treatment alone results in surfaces with activity comparable to those prepared with the more hazardous solutions.

The surface activity of chips that had been stored wet or dry for 1 wk was comparable to surface activity measured immediately after immobilization (Fig. 1, column sets 4-5). It has been reported that immobilized antibody surfaces displayed no loss of activity after 28 d at 4°C in PBS-azide (*14*) or even after 95 d at 6°C in Tris-HCl buffer (*24*); but, a refrigerated wet storage/transport protocol is expensive and inconvenient. Sucrose treatment and dehydration, directly adapted from an ELISA plate protocol, was effective at maintaining surface activity for at least 7 d. Maximum storage times and storage temperature tolerances still need to be defined; use of established ELISA protocols is recommended to facilitate such investigations.

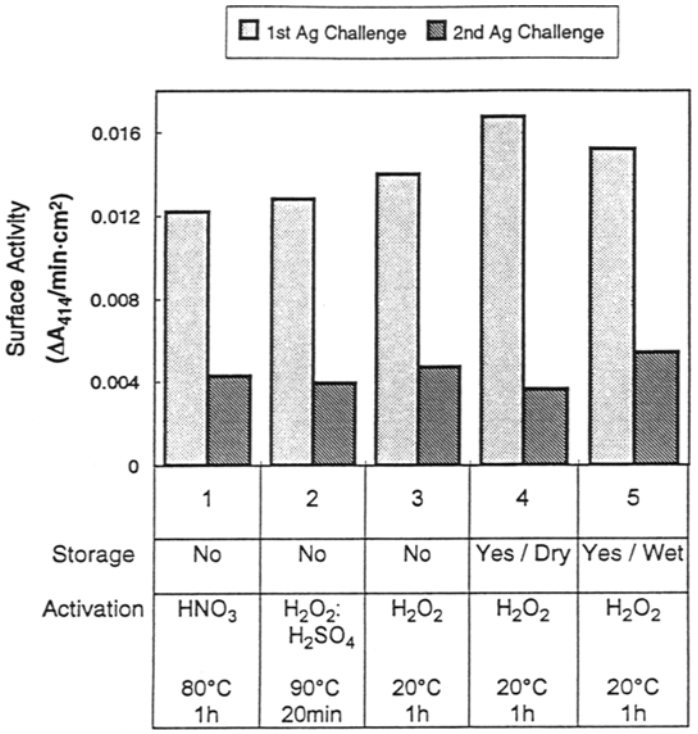


Fig. 1. Column sets 1–3: Comparison of surface activity obtained using one of three chemical treatments for surface cleaning and silanol activation; (1) 1 h soak in 80°C nitric acid; (2) 20 min soak in 5:1 $H_2SO_4$:$H_2O_2$; (3) 1 h soak in $H_2O_2$. Column sets 3–5: Surface activity comparison among chips that were: (3) challenged with antigen immediately after antibody immobilization; (4) stabilized with 2% (w/v) sucrose, dehydrated at 37°C for 1 h, stored for 7 d at ~100 mtorr; (5) stored for 7 d in PBS-azide at 4°C. All chips were $SiO_2$ for these comparisons. Chips were soaked in 0.1*N* acetic acid for 5 min, rinsed, then stored in PBS-azide at 4°C for 1–3 d between sequential antigen challenges.

## Multiple Antigen Challenges and Nonspecific Binding

Preparation of glutaraldehyde-treated and nontreated surfaces enabled a comparison of the amount of surface activity owing to covalently bound antibody to the amount of surface activity owing to nonspecifically adsorbed antibody. Samples that received glutaraldehyde represent activity caused by covalently linked *plus* nonspecifically adsorbed antibody; samples that did not receive glutaraldehyde treatment represent adsorbed antibody only. The surfaces are not identical, but the comparison is still useful. The activity measured after the first challenge with antigen (Fig. 2) demonstrates that the adsorbed antibody load on the silanized only surfaces is

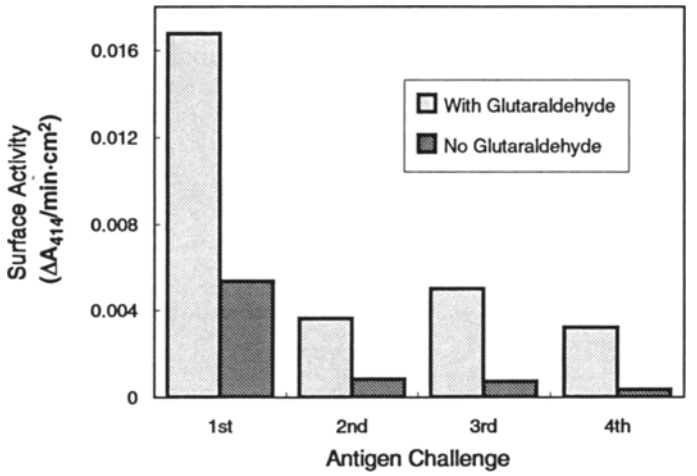


Fig. 2. Surface activity comparison between chips that received all chemical treatments of the immobilization procedure and chips that received all chemical treatments except glutaraldehyde. All chips were $SiO_2$ for this comparison. Chips were soaked in 0.1*N* acetic acid for 5 min, rinsed, then stored in PBS-azide at 4°C for 1–3 d between sequential antigen challenges.

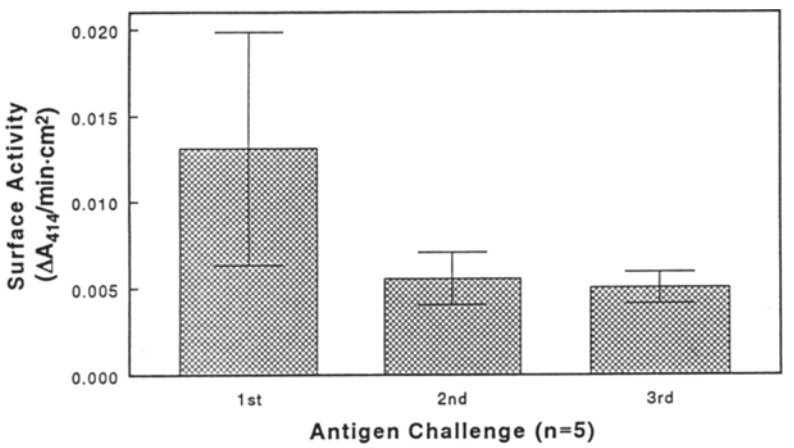


Fig. 3. Surface activity (mean ± sd) of five separate immobilization trials. All chips were $SiO_2$ for this comparison. Immobilization conditions were identical for all chips. Chips were soaked in 0.1*N* acetic acid for 5 min, rinsed, then stored in PBS-azide at 4°C for 1–3 d between sequential antigen challenges.

less than that of the total (adsorbed plus covalent) load on the glutaraldehyde-treated surfaces. This agrees well with the results of Williams and Blanch (*14*), who reported that adsorption loadings after silanization were greater than total loadings with glutaraldehyde unless immobilization was performed in the presence of Tween 60.

Figure 2 also indicates a large decrease in the activity of both the glutaraldehyde and nonglutaraldehyde treated samples after the first antigen

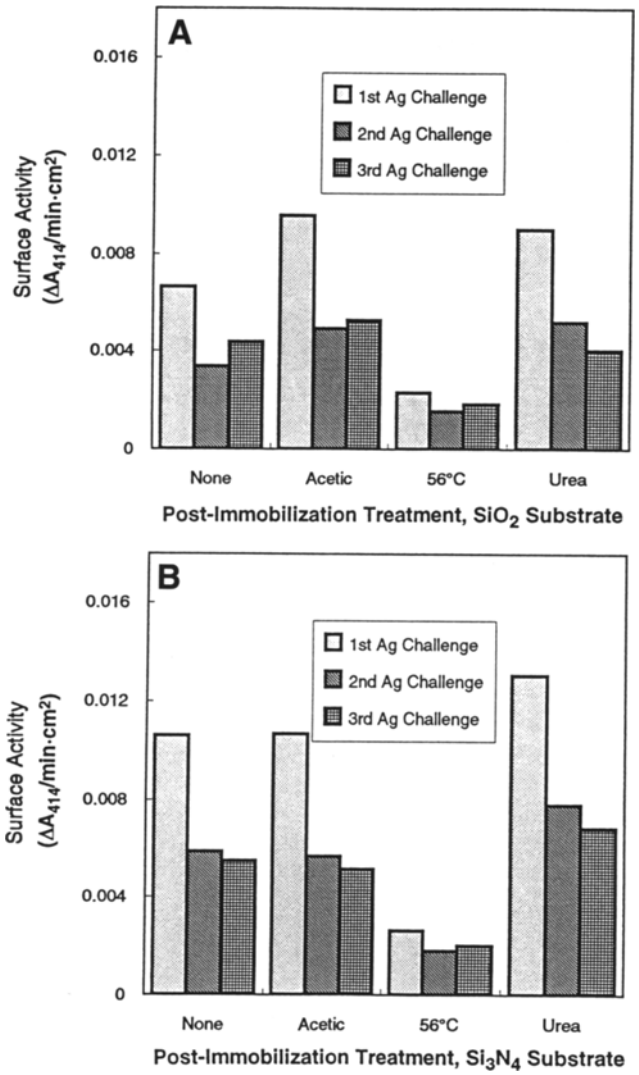


Fig. 4. (**A**) Surface activity comparison among $SiO_2$ chips that received one of a variety of postimmobilization treatments to reduce nonspecifically bound antibody. Chips received the postimmobilization treatment only prior to the first challenge with antigen. Acetic—5 min soak in 0.1*N* acetic acid; 56°C—4 h soak in 56°C PBS; Urea—5 min soak in 6*M* urea. All chips were soaked in 0.1*N* acetic acid for 5 min, rinsed, then stored in PBS-azide at 4°C for 1-3 d between sequential antigen challenges. (**B**) The same surface activity comparison among $Si_3N_4$ chips.

challenge. However, on subsequent antigen challenges, nonglutaraldehyde treated samples gradually approached zero activity, whereas glutaraldehyde treated samples maintained a relatively constant activity. Additional evidence that glutaraldehyde treated surfaces maintained a constant activity after the first antigen challenge is provided by Figs. 3-6.

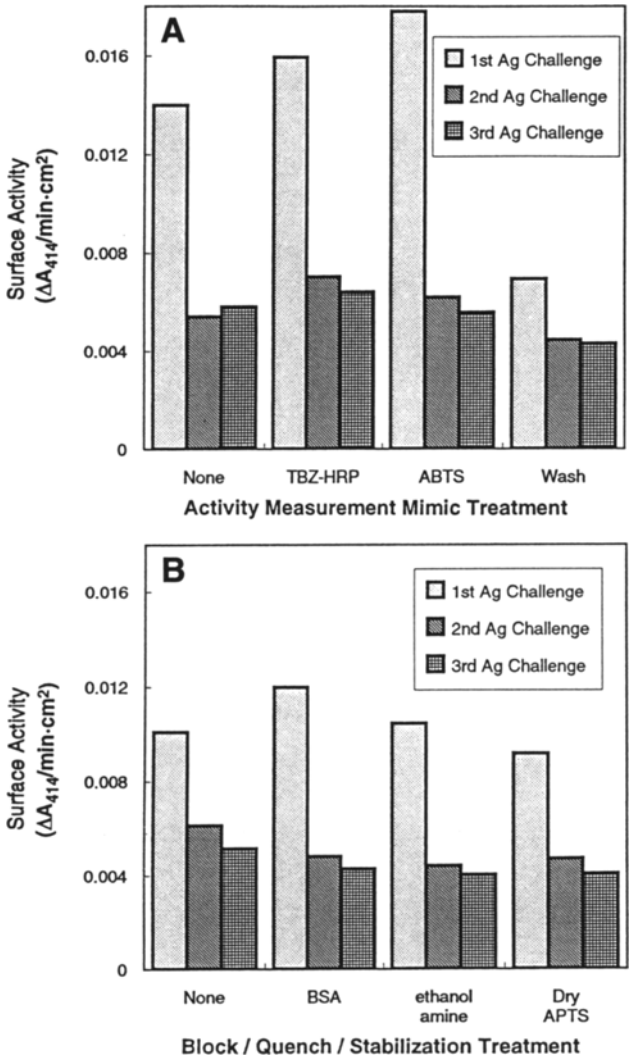


Fig. 5. (**A**) Surface activity comparison among chips that received post-immobilization treatments that reproduced the chemical treatments used during a surface activity measurement. Chips received the postimmobilization treatment only prior to the first challenge with antigen. All chips were $SiO_2$ for this comparison. TBZ-HRP—1 h soak in 100 ng/mL TBZ-HRP in PBS containing 10 mg/mL BSA followed by 5 min soak in 0.1*N* acetic acid; ABTS—1 h soak in 1 m*M* ABTS and 6.7 m*M* $H_2O_2$ in 60 m*M* sodium citrate buffer, pH 4.2; Wash—overnight soak at room temperature in PBS-Tween 20 with agitation. Chips were soaked in 0.1*N* acetic acid for 5 min, rinsed, then stored in PBS-azide at 4°C for 1-3 d between sequential antigen challenges. (**B**) Surface activity comparison among chips that received postimmobilization treatments designed to prevent secondary protein adsorption or stabilize covalently bound protein. Chips received the postimmobilization treatment only prior to the first challenge with antigen. All chips were $SiO_2$ for this comparison. BSA—1 h soak in PBS containing 10 mg/mL bovine serum albumin; ethanolamine—1 h soak in 0.2*M* ethanolamine pH adjusted to 9.0 with 12*N* HCl; dry APTS—chips were dehydrated overnight in a convection oven at 110°C after silanization.

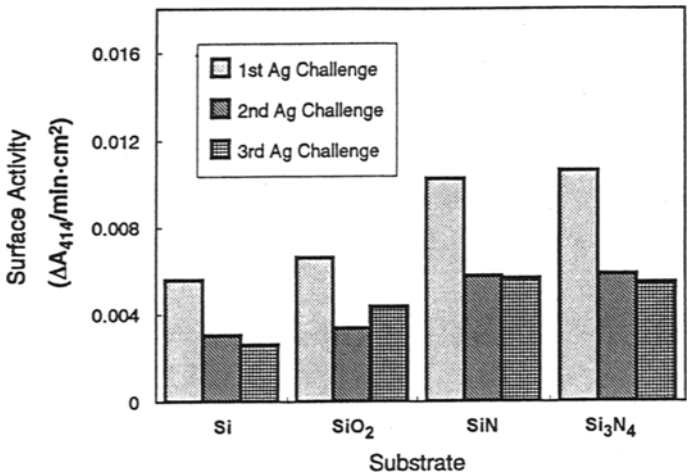


Fig. 6. Surface activity comparison among the four substrate materials investigated. Si—bare silicon surface; $SiO_2$—silicon substrate with ~100 nm $SiO_2$; SiN—silicon substrate with ~100 nm silicon-rich silicon nitride; $Si_3N_4$—silicon substrate with ~100 nm stoichiometric silicon nitride. Chips were soaked in 0.1*N* acetic acid for 5 min, rinsed, then stored in PBS-azide at 4°C for 1-3 d between sequential antigen challenges.

This result suggests that the acetic acid wash between antigen challenges served to remove adsorbed antibody as well as bound antigen.

There was wide scatter in the initial surface activity of chips that were prepared under identical conditions but for different experiments (e.g., 1st challenge, $SiO_2$, Figs. 1, 2, 4–6). Figure 3 shows the large error in initial surface activity when several chip sets with identical preparation conditions were averaged. However, the error in surface activity decreases significantly for the second and third antigen challenges (Fig. 3, cols. 2 and 3). This pattern is further evidence that the initial surface activity is caused by the combination of covalently linked and nonspecifically adsorbed antibody, whereas subsequent activity is primarily owing to covalently bound antibody. The deviation in initial activity was likely caused by widely varying amounts of adsorbed antibody for each chip set; secondary and tertiary activity measurements displayed a constant activity since adsorbed antibody had been removed and a reproducible covalently bound antibody load remained.

Since the acetic acid wash was assumed to be the treatment responsible for desorption of adsorbed antibody, a chip set was treated with acetic acid prior to the first challenge with antigen (Fig. 4A and B, column set 2). Surprisingly, the initial surface activity was equal to (Fig. 4B) or greater than (Fig. 4A) the control set that received no acetic acid wash. However, the acetic acid treated chips displayed the same behavior as all previous chip sets; subsequent challenges with antigen resulted in a decreased but relatively constant surface activity. The increased activity of the acetic

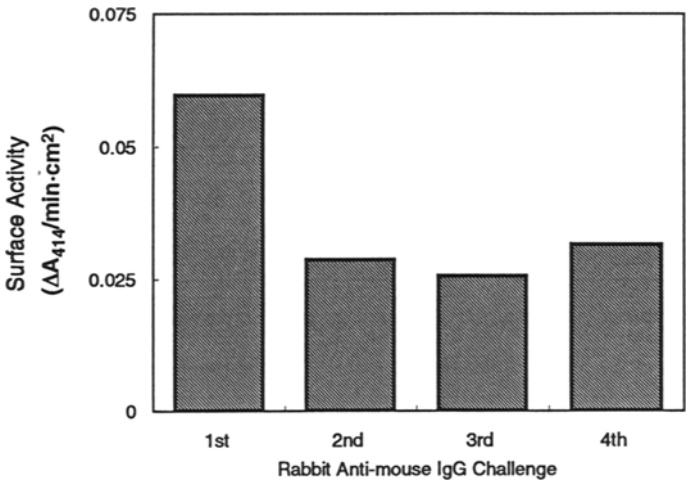


Fig. 7. Surface activity comparison among chips that were challenged with Rabbit anti-mouse IgG-HRP rather than the usual TBZ-HRP. All chips were $SiO_2$ for this comparison. Chips were soaked in 0.1*N* acetic acid for 5 min, rinsed, then stored in PBS–azide at 4°C for 1–3 d between sequential antigen challenges.

acid treated surfaces may be similar to previously reported intentional attempts to enhance antibody binding by low-pH treatments (acid shock) (*25*). This possible relationship was not pursued. Rather, additional experiments were performed to determine which treatments were responsible for protein desorption.

During a surface activity measurement, the surface is treated consecutively with hapten–enzyme conjugate, ABTS substrate in citrate buffer, and finally acetic acid. Any of these chemical treatments could have been responsible for antibody desorption. Therefore, separate chip sets were pretreated with each of these solutions prior to the surface activity measurement (Fig. 5A). (The chips treated with hapten–enzyme were subsequently treated with acetic acid before the first activity measurement.) None of the treatments resulted in a decreased initial surface activity relative to the control chip set; and, the second surface activity measurement displayed the usual activity decrease (Fig. 5A). Since no single component of the activity measurement treatments could be identified as the agent responsible for antibody desorption, a probable explanation is that the entire process of surface activity measurement with its multiple wash and rinse steps led to gradual desorption of nonspecifically bound antibody. The chip set that was soaked in PBS-Tween 20 overnight (Fig. 5A, column set 4) displayed a lower initial activity and smaller activity decrease after primary challenge with antigen, further supporting this gradual desorption explanation.

The best evidence that the decreased activity was caused by desorption of nonspecifically bound antibody (rather than antibody inactivation) is provided by Fig. 7. Challenging the chips with RαMIgG-HRP gave a

relative measure of amount of immobilized antibody rather than activity of immobilized antibody. The activity measured with RαMIgG-HRP decreased in parallel with the surface activity measured with TBZ-HRP.

To address the possiblity that the surface activity decrease was caused by loss of covalently bound antibody owing to hydrolysis of the siloxane linkage, a chip set that was dehydrated after APTS treatment was prepared. Dehydration has been recommended to increase the stability of APTS treated surfaces (*15*). The surface activity of the dehydrated samples was the same as that of control chips (Fig. 5B, column set 4). This result indicates that the loss of activity after primary antigen challenge was not avoided by dehydration after APTS treatment and was probably not owing to antibody loss by siloxane hydrolysis.

Additional post-immobilization treatments were investigated to define conditions that would remove adsorbed antibody so that reproducible initial surface activities could be obtained. Treatment with 6*M* urea had little or no effect on the initial surface activity (Fig. 4A and B, column set 4). Curing at 56°C however, reduced dramatically the initial surface activity. This treatment was adopted from a recent review of protein immobilization (*15*). The temperature seems high and rather arbitrary, but it is assumed these conditions were adapted from heat treatments defined to inactivate complement while still maintaining IgG activity (*26*). Curing was the only treatment that yielded a surface whose initial surface activity was equivalent to secondary and tertiary activities (Fig. 4A and B). It should be noted though that the heat treatment resulted in surface activity approximately one half the final activity of control surfaces. It is not clear whether the heat treatment caused an additional activity decrease by removing a greater amount of adsorbed antibody or by inactivating some of the immobilized antibody.

Chips that received treatments to minimize secondary protein adsorption displayed surface activities essentially the same as that of control chips (Fig. 5B). Since the TBZ-HRP solution contained 1% BSA, control chips also received a blocking solution. Though control chips are blocked and challenged with antigen concurrently, this blocking was sufficient. Pretreatment with BSA or quenching with a low molecular weight amine did not affect significantly the surface activity.

## Substrate Comparison

Upon exposure to air, the surface layer of a bare silicon surface oxidizes forming a "native oxide" (*27*) and the surface layers of a silicon nitride film oxidize to $SiO_2$ owing to the oxide's greater thermodynamic stability (*28*). Therefore, a surface silicon dioxide layer is present for all films investigated and little difference among the protein loads immobilized to the various surfaces was expected or observed (Fig. 6). The bare silicon, silicon-rich silicon nitride and stoichiometric silicon nitride chips

Table 1
Enzyme Turnover Number for Conjugated and Nonconjugated HRP

| Enzyme solution | Activity, (molecules substrate converted) / molecule enzyme·s) |
|---|---|
| TBZ-HRP conjugate | 89.4 ± 21.1 ($n$ = 4) |
| "Fresh" HRP | 469.2 ± 12.4 ($n$ = 3) |
| Stored HRP | 97.3 ± 15.9 ($n$ = 4) |

all displayed behavior similar to the silicon dioxide chips, high initial activity followed by decreased but stable secondary and tertiary activity (Fig. 6). Figure 6 indicates that the activity of the silicon nitride surfaces was greater than that of the Si or $SiO_2$ surfaces. However, Fig. 3 indicates that the increased activity of the nitrides did not exceed the experimental error of the measurements. Also, little difference is noted between the $SiO_2$ and $Si_3N_4$ substrates when comparing postimmobilization treatments, especially after curing (Fig. 4A and B). This result suggests that nonspecific adsorption to the nitride surfaces may be greater, but the density of covalently linked antibody is equivalent for the oxide and nitride. For a more quantitative comparsion, estimation of the surface silanol density of the oxide and nitride surfaces for each of the surface activation treatments (e.g., with a surface analysis technique such as grazing angle X-ray photoelectron spectroscopy) is recommended.

## Enzyme Activity and Final Antibody Loading

Enzyme activity was measured so that surface activity measurements ($\Delta A_{414}$/min·cm$^2$) could be translated into an estimate of antibody loading (antibody molecules/cm$^2$). Assuming an enzyme molecular weight of 40,000, the supplier estimates enzyme turnover (25°C, pH 5.0) to be approx 660 molecules substrate converted per molecule enzyme-second. Measurements of the TBZ-HRP activity (Table 1) were much lower than expected even considering the lower pH (4.2) of our substrate solution. Therefore, the activity of the HRP alone was also measured (Table 1). When measured immediately after solubilizing the HRP, activity is comparable to the supplier's claims. However, when activity was remeasured after storage for 8 wk at conditions typically used for conjugate storage (4°C, ~ 100 μg/mL), the HRP activity had decreased to approximately the same level as measured for the TBZ-HRP conjugate. This result implies that the decreased activity of the conjugate is not owing to the conjugation procedure nor to some steric hindrance caused by the hapten. Rather, the decreased activity is characteristic of the enzyme in dilute solution. Products specifically designed to stabilize HRP in solution have been marketed (*29*).

Table 2
Ellipsometry Measurements After Each Step of Immobilization Procedure

| Sequential chemical treatments | Thickness (nm) R.I. fixed at 1.46 ($n$ = 16) |
|---|---|
| Initial $SiO_2$ Film | 114.9 ± 1.1 |
| +APTS treatment | 114.9 ± 0.9 |
| +Glutaraldehyde treatment | 115.8 ± 1.8 |
| +Contact with αTBZ antibody | 124.4 ± 1.1 |
| +Contact with TBZ-BSA | 128.2 ± 2.9 |

Using the enzyme turnover number measured for the TBZ-HRP conjugate and assuming one TBZ-HRP molecule bound per antibody molecule, the typical residual surface activity of .004 $\Delta A_{414}$/min·cm$^2$ corresponds to an antibody loading of approximately 2 × $10^{11}$ antibody molecules/cm$^2$. It has been estimated that the densest monolayer packing of antibody on a surface results in a surface loading of 1–2 × $10^{12}$ antibody molecules/cm$^2$ (*30*). Thus, the stable and reproducible surface activity measured after a second or later challenge with antigen is approximately one fifth of a monolayer and is in general agreement with the results of other investigators (*14,31*).

## Ellipsometry

The ellipsometry results (Table 2) indicate no thickness change owing to the APTS treatment. This result may be due to the fact that the refractive index was fixed for the measurement. The slight increase in film thickness expected for an aminosilane monolayer (0.5–1.0 nm) may be offset by the change in refractive index that the additional layer caused. In previous work (*32*), large changes in thickness after silane treatment have been associated with deposition of polymolecular siloxane films. Therefore, a small or zero thickness change after APTS treatment suggests deposition of the desired monomolecular layer. Monolayers are typically generated when using trifunctional silanes in aqueous solution. Glutaraldehyde treatment produced a thickness change greater than might be expected for a monolayer of such a small molecule. This result supports the previously proposed mechanism (*33*) that glutaraldehyde forms a stable polymeric layer via aldol condensations resulting in several glutaraldehyde molecules per amine function. Thickness change after contact with TBZ specific antibodies (8.6 nm) corresponds well with the typical models of IgG as an oblong molecule approx 3 nm × 15 nm (*30*). The 8.6 nm thickness change along with the tight standard deviation (1.1 nm) also suggests that a monomolecular layer of IgG was deposited rather than polymeric clumps of antibody. Finally, the thickness change after challenge with TBZ-BSA (3.8 nm) is as expected for a molecule the size of BSA but the standard deviation of the measurement was large. It is

possible that the BSA conjugate polymerized on the chip surface or that binding of a large conjugate such as TBZ-BSA at one site sterically hindered binding at neighboring sites. In either case, a nonuniform TBZ-BSA layer would be bound and hence the standard deviation of the measurement would be large. The larger TBZ-BSA conjugate rather than TBZ-HRP was used for this measurement to increase the likelihood of detecting a measurable thickness change. Ellipsometry measurements with TBZ-HRP were not performed.

## SUMMARY

Thiabendazole-specific monoclonal antibodies were successfully immobilized to silicon, silicon dioxide, and silicon nitride substrates. Little difference was noted among the antibody loading achieved on the various substrates. The surfaces could be stabilized and dehydrated or stored wet without affecting antibody activity. After elution of bound antigen with 0.1*N* acetic acid, the antibody coated surfaces could be reused. A stable and reproducible surface loading of $2 \times 10^{11}$ antibodies/cm$^2$ was obtained only after surfaces received postimmobilization treatments that presumably removed nonspecifically bound antibody. Soaking in PBS at 56°C (curing) was the most effective antibody desorption treatment investigated; however, the temperature may be more severe than necessary. Curing or an exhaustive postimmobilization wash with PBS and detergent is recommended so as to obtain reusable antibody coated surfaces that display reproducible activity. Results indicated that the primary issue continues to be insuring that the antibodies attached to the surface are actually covalently linked rather than nonspecifically adsorbed.

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