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# A Quantitative Assessment of Heterogeneity for Surface-Immobilized Proteins

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**Many biotechnological applications use protein receptors immobilized on solid supports. Although, in solution, these receptors display homogeneous binding affinities and association/dissociation kinetics for their complementary ligand, they often display heterogeneous binding characteristics after immobilization. In this study, a fluorescence-based fiber-optic biosensor was used to quantify the heterogeneity associated with the binding of a soluble analyte, fluorescently labeled trinitrobenzene, to surface-immobilized monoclonal anti-TNT antibodies. The antibodies were immobilized on silica fiber-optic probes via five different immobilization strategies. We used the Sips isotherm to assesses and compare the heterogeneity in the antibody binding affinity and kinetic rate parameters for these different immobilization schemes. In addition, we globally analyzed kinetic data with a two-compartment transport–kinetic model to analyze the heterogeneity in the analyte–antibody kinetics. These analyses provide a quantitative tool by which to evaluate the relative homogeneity of different antibody preparations. Our results demonstrate that the more homogeneous protein preparations exhibit more uniform affinities and kinetic constants.**

Due to the high specificity of biomolecular interactions, surface-immobilized proteins have found wide use in many biotechnological applications. For example, in affinity chromatography, protein receptors immobilized on porous particles are used to separate a ligand from dilute mixtures. Immobilized proteins are also a key component in the interfacial recognition layer of many biosensors. In these devices, proteins such as enzymes or antibodies are immobilized directly on a solid substrate or within a porous matrix and used to detect the presence of a target substrate or antigen in a variety of sample media. These devices are now popular tools for detecting a wide array of analytes in medical diagnostics, environmental monitoring, and defense applications.<sup>1,2</sup>

A principal problem with the above devices is the loss of biological activity that often accompanies the immobilization of

the protein component. When proteins are physically adsorbed or covalently attached to a solid support, they typically display smaller binding affinities and specific binding capacities than their soluble counterparts.<sup>3,4</sup> These trends are largely attributed to steric blockage of the receptor's active sites by the supporting matrix. In many cases, a protein's amine groups are used to covalently immobilize it to a solid support. Since amine groups are scattered throughout the structures of most proteins, the receptor is randomly immobilized in many different orientations. In many of these configurations, binding activity is decreased because the receptor's binding sites are near the surface and thus hindered from binding to the ligand. Besides steric effects, it has also been shown that the chemical microenvironment near the surface can impact activity.<sup>5,6</sup> In addition, immobilization may also cause conformational changes or even denaturation.<sup>7</sup>

Upon immobilization, not only does protein activity decrease, it also becomes heterogeneous. In solution, monoclonal antibodies, many enzymes, and other receptors exhibit uniform kinetic rate constants and binding affinities. Despite this, they often possess nonuniform kinetic and thermodynamic properties after surface immobilization. Rather than having a unique affinity and a single set of association and dissociation rate constants, most biomolecular interactions are typically described by a distribution of these values. Such heterogeneity arises because steric effects, conformational changes, and the chemical microenvironment can influence each immobilized biomolecule to a different extent. This may happen, for example, if the point of attachment, and thus the protein orientation, differs from one molecule to the next or if the chemical microenvironment near the supporting matrix is itself spatially nonuniform.

Both heterogeneity and the decreased binding activities that accompany immobilization are detrimental to biosensors and other protein-based devices. These effects limit the sensitivity and the reproducibility of surface-immobilized protein films. In fact, several studies suggest that randomness in the antibody's spatial orientation limits sensor sensitivity.<sup>2,8</sup> Previously, we also found that it is difficult to accurately predict biosensor detection kinetics with

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a heterogeneous immobilized antibody population.<sup>9</sup> Heterogeneity thereby complicates the design of biosensor devices because the kinetic and thermodynamic parameters that describe detection are characterized by an unknown distribution of values rather than known constants.

Consequently, there is considerable interest in maximizing the biological activity and eliminating the heterogeneity associated with immobilized proteins. So far, researchers have focused on creating films of uniformly oriented protein receptors in an attempt to increase the accessibility of their binding sites. By uniformly orienting the receptors, it is also hoped that any steric effects that accompany immobilization will affect all of them in the same way and thus reduce heterogeneity. The general strategy for achieving this has been to bind the protein via unique surface amino acids rather than random amine groups on the protein's exterior.<sup>3,7,10–12</sup> In the case of antibodies, for example, several coupling chemistries have been devised to couple the carbohydrate moiety on the antibody's Fc region to the substrate, thus orienting the antibody combining sites away from the supporting solid matrix.<sup>3,13</sup> Others have attempted to create similar films with Fab' fragments.<sup>4</sup> The Fab's binding site is positioned away from the surface by coupling the sulfhydryl moieties, located on the fragment's C-terminus, to the substrate. In addition to antibodies, oriented films have been constructed with other proteins by genetically engineering unique attachment points into the protein's structure.<sup>5–7</sup>

A myriad of studies demonstrated the efficacy and compared the features of the above surface-immobilization strategies.<sup>8,13–16</sup> So far, attention has largely focused on evaluating the biological activities associated with these different methods. Several studies confirmed that orienting antibodies on the surface does increase specific antibody activity.<sup>2,8,13</sup> By comparison, a limited amount of work has been done to characterize the heterogeneity of surface-immobilized proteins. Previous investigations demonstrated methods to assess the heterogeneity in the receptor–ligand affinity and binding kinetics.<sup>17–19</sup> Other researchers indirectly studied heterogeneity by measuring the spatial orientation of surface-bound heme proteins.<sup>12,20</sup> These measurements were performed with several immobilization chemistries and thus illustrate how the immobilization chemistry can impact protein orientation. While the orientation is related to heterogeneity, such orientation measurements do not definitively indicate how the immobilization chemistry impacts the uniformity of the protein's kinetic and thermodynamic properties.

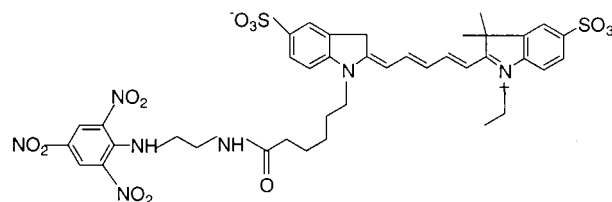


Figure 1. Chemical structure of the analyte cyanine-5-ethylenediaminetrinitrobenzene (Cy5-TNB).

In this study, we directly probed the relationship between the protein immobilization chemistry and the heterogeneity in the protein's affinity and binding kinetics. We used a fiber-optic biosensor to assess the heterogeneity associated with the binding of cyanine-5-ethylenediaminetrinitrobenzene (Cy5-TNB), a fluorescently labeled analogue of TNT (Figure 1), to surface-immobilized monoclonal anti-TNT antibodies. We immobilized anti-TNT antibodies via five different immobilization methods. For these methods, we assessed the heterogeneity in the analyte–antibody binding affinity and kinetic rate parameters. Like many previous investigations, we also evaluated the antibody activity for each of the immobilization chemistries. We then correlated these observations with the corresponding results from our heterogeneity analysis. Quantitative analyses of both antibody heterogeneity and immunological activity identified immobilization chemistries that yield protein films with both high biological activity and homogeneous binding characteristics. These immobilization methods can be used to construct highly sensitive and reproducible protein films for use in biosensors and other applications that use surface-immobilized antibodies. Importantly, our studies demonstrate that the analytical methods described in this work can provide quantitative design criteria for the fabrication of immobilized protein sensors.

## MATERIALS AND METHODS

**Reagents.** Fiber-optic immunoassay experiments were completed with either monoclonal anti-TNT antibodies (Clone A1.1.1, Strategic Biosolutions, Ramona, CA) or polyclonal goat IgG (Sigma-Aldrich Corp., St. Louis, MO). Cy5-TNB was kindly provided by the U.S. Naval Research Laboratory (Washington, D.C.). The heterobifunctional cross-linkers *N*-succinimidyl 4-maleimidobutyrate (GMBS) and biotin maleimide (BM) were obtained from Sigma-Aldrich. The protein modification reagents biotin-LC-hydrazide and 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylhydrazide HCl (M<sub>2</sub>C<sub>2</sub>H) were purchased from Pierce (Rockford, IL). Bovine serum albumin (BSA), protein G, (3-mercaptopropyl)trimethoxysilane (MPTS), and ethanolamine were acquired from Sigma-Aldrich. Streptavidin was purchased from Calbiochem (San Diego, CA). The following additional reagents were supplied by Fisher Scientific (Pittsburgh, PA): toluene, sodium azide, glycerol, dimethyl sulfoxide (DMSO), and Tween-20 (T-20).

**Antibody Immobilization Strategies.** We investigated the immobilization chemistries illustrated in Figure 2. First, antibodies were randomly oriented on the fiber surface through their amine groups. In the remainder of the immobilization schemes, we attempted to uniformly orient the antibody's binding sites away from the solid support by localizing the point of surface attachment on the protein to the antibody's Fc region. This was done by either

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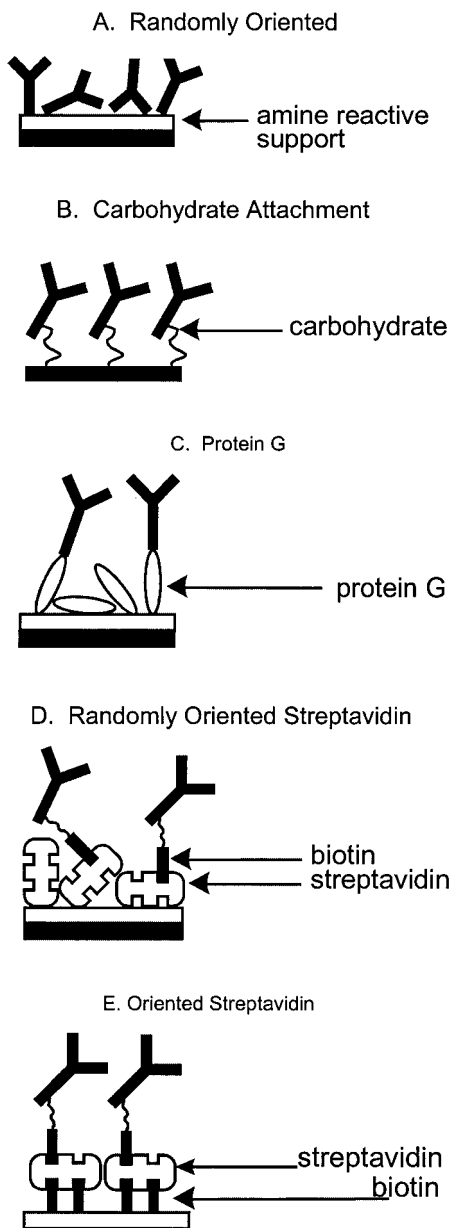


Figure 2. Monoclonal anti-TNT antibodies immobilized on silica fiber-optic probes via five different strategies. Antibodies were randomly oriented on the surface via their primary amine groups (A) or through the carbohydrate moiety on the antibody's Fc domain (B). Alternatively, antibodies were immobilized through an intervening protein layer such as an unoriented protein G film (C) or randomly oriented (D) and uniformly oriented (E) streptavidin layers.

directly attaching the carbohydrate moiety to the silica surface or linking the antibody's Fc region to another protein already immobilized on the sensor surface. For example, one common approach that we tested is to bind antibody to the Fc receptor protein G. Another alternative that we investigated is to modify the antibody's carbohydrate with biotin and to then bind the modified antibody to immobilized streptavidin. With these latter two approaches, protein G and streptavidin were immobilized via their amine groups and were thus not aligned on the sensor surface. To investigate how the orientation of the intervening protein layer might affect antibody heterogeneity, we also immobilized antibodies on uniformly oriented streptavidin films. These films were prepared by binding streptavidin to biotin

functionalized fiber-optic probes. The streptavidin should be uniformly aligned since it attaches to the surface via one of its four, symmetrically arranged biotin binding sites rather than random amine groups on its exterior.

**Fiber-Optic Biosensor and Fiber Preparation.** All experiments were performed with the Analyte 2000 biosensor (Research International, Woodinville, WA) described previously.<sup>2</sup> The Analyte 2000 is equipped with a 635-nm diode laser that is capable of exciting Cy5 fluorescence. Excitation light from the laser is coupled to a fiber-optic probe. As light propagates along the length of the fiber via total internal reflection, fluorescent molecules near the fiber surface are excited. The emitted light, which has a longer wavelength than the excitation light, is coupled back through the fiber so that its intensity can be quantified by an attached fluorometer.

The preparation of the fiber-optic probes has been described extensively.<sup>21–23</sup> Briefly, the fiber-optic probes were constructed from 600- $\mu$ m-diameter, plastic-clad, silica fibers (Quartz Products, Tuckerton, DE). The sensing region of the fiber was formed by removing the plastic cladding that surrounds the last 12.5 cm of the probe. This region was then tapered with hydrofluoric acid to a diameter of 100  $\mu$ m at the fiber's distal end. Antibodies were then covalently attached to the fiber probes. Five different immobilization chemistries were used to immobilize the monoclonal anti-TNT antibodies. The details of these surface-coupling schemes are described below. After antibody immobilization, each fiber was sealed within a flow chamber constructed from a 100- $\mu$ L glass capillary tube capped by T-connectors at both ends.

**Immobilization via Amine Groups.** Antibodies were directly immobilized to the fiber surface by following the procedure outlined by Bhatia et al.<sup>24</sup> Briefly, tapered fibers were placed in boiling water for 15 min to enhance the formation of surface hydroxyl groups. After the fibers were air-dried, they were treated with a 2% solution of MPTS in toluene for 30 min and then dipped in a 2 mM solution of GMBS in ethanol for 1 h. After the MPTS covalently attaches to the fiber surface, its thiol group reacts specifically with the maleimide moiety on the GMBS cross-linker. This activates the fiber surface with succinimide residues that can react with amine groups of an antibody or other protein to form stable amide bonds. After modification with GMBS, the succinimide-activated fibers were rinsed three times with water and then stored overnight in a 0.05 mg/mL anti-TNT Mab solution in phosphate-buffered saline (PBS; 0.1%  $\text{NaN}_3$ , pH 7.4).

**Immobilization via the IgG Carbohydrate Moiety.** Antibodies were also directly coupled to the fiber surface via their carbohydrate moiety as previously described.<sup>15,25</sup> Before coupling to the surface, antibodies were treated with sodium *m*-periodate and  $\text{M}_2\text{C}_2\text{H}$ . Antibodies were incubated in a 10 mM solution of sodium *m*-periodate dissolved in cold NaOAc buffer (0.1 M sodium acetate buffer, pH 5.5). This mixture was placed in the dark for

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30 min at room temperature. The sodium *m*-periodate oxidizes the carbohydrate's vicinal diols to form aldehyde groups. The oxidation reaction was quenched by adding 5  $\mu$ L of glycerol to the reaction mixture. The antibody was then separated from the unreacted sodium *m*-periodate and glycerol with an Amicon concentrator with a YM-30 membrane (Millipore, Bedford, MA). Next, a 50 mM solution of  $M_2C_2H$  was prepared in DMSO. This solution was added to the modified antibody so that the final concentration of  $M_2C_2H$  cross-linker was 5 mM. The resulting solution was mixed well and then incubated for 2 h in the dark. In this step, the cross-linker's hydrazide moiety reacts with the carbohydrate aldehydes to form stable, covalent hydrazone bonds and leaves reactive maleimide groups attached to the carbohydrate. After the antibody was separated from unreacted  $M_2C_2H$  with an Amicon concentrator, the modified antibody was loaded onto the fiber surface. Fiber-optic probes were activated with MPTS as described above and then exposed overnight to a 0.05 mg/mL solution of the modified antibody in PBS. Antibodies attached to the surface covalently by forming stable thioether bonds between their maleimide groups and the thiols on the fiber surface.

**Immobilization via Protein G.** Protein G was attached to fiber-optic probes with the amine reactive chemistry described above. After treatment with GMBS, the fiber probes were immediately incubated for 1 h in a 0.05 mg/mL solution of protein G in PBS. To quench any unreacted succinimide residues that did not couple to protein G, the probes were rinsed in PBS and then dipped in a 1 M solution of ethanolamine, pH 8.5 for 15–30 min. The fiber probes were rinsed with PBS again and finally incubated overnight in a 0.05 mg/mL solution of the anti-TNT antibody in PBS.

**Immobilization via Streptavidin.** A procedure similar to that used to attach  $M_2C_2H$  to the anti-TNT antibody was used to biotinylate the antibody's carbohydrate moiety. The anti-TNT antibody was treated with sodium *m*-periodate as described above. However, instead of then treating the antibody with  $M_2C_2H$ , the modified antibody was reacted for 2 h with a 5 mM solution of biotin-LC-hydrazide in NaOAc buffer. The hydrazide group on the biotin-LC-hydrazide reacts with the oxidized carbohydrate, thus labeling the antibody with biotin. The antibody was purified from the reaction mixture with an Amicon concentrator. The biotinylated anti-TNT antibody was then loaded onto the fiber-optic probes.

Streptavidin-coated fiber-optic probes were prepared via two different methods. One set of streptavidin-modified fibers was prepared by following the amine reactive procedure described above. To construct oriented streptavidin layers, the surfaces of another set of fibers were activated with biotin. To do this, fiber probes were dipped in a 2 mM solution of BM after treatment with MPTS. The fiber surface was coated with biotin as the maleimide group of BM reacted with the thiol-terminated silane. After 1 h, the biotin-coated fibers were rinsed with water and then incubated in a 0.05 mg/mL solution of streptavidin in PBS overnight. Finally, to immobilize the anti-TNT antibodies, streptavidin-coated fiber probes were stored overnight in a 0.05 mg/mL solution of the biotinylated antibody in PBS.

**Radiolabeling Measurements.** To evaluate the immunological activity associated with each of the immobilization strategies

in Figure 2, it was necessary to measure the antibody surface density for each immobilization scheme. We did this by employing each of the above immobilization protocols to covalently couple radiolabeled goat IgG to glass cover slips. Cover slips (Corning, Corning, NY) measuring  $2.2 \times 4.0$  cm were cut to a width of 1.5 cm and then cleaned in a warm 1:1:1 solution of deionized water, HCl, and  $H_2O_2$ . They were then rinsed with water and sonicated for 30 min. After being rinsed again, the cover slips were dried under a stream of nitrogen. Following the previously described procedures, the cover slip surfaces were activated with thiol groups, succinimide groups, streptavidin, or protein G. Depending on the resulting surface functionality, the cover slips were then exposed overnight to a 0.05 mg/mL solution of unmodified, biotinylated, or  $M_2C_2H$ -treated goat IgG in PBS. All antibodies were labeled with  $^{125}I$  using Iodobeads (Pierce) as described previously.<sup>26</sup> Biotinylation or treatment with  $M_2C_2H$  was always completed before radiolabeling the antibody. After incubation, the cover slips were rinsed and then shaken in PBS for 1 h to remove any nonspecifically bound antibody. After rinsing in PBS again, the antibody surface density was determined by counting each of the cover slips with a Beckman scintillation counter.

**Assessing Heterogeneity in the Analyte–Antibody Binding Affinity.** The Sips isotherm was used to assess the heterogeneity in the antibody affinity. This approach has been applied to evaluate the heterogeneity in antigen–antibody binding in solution.<sup>27</sup> More recently, it has also been applied to study the heterogeneity associated with the displacement of antigen from surface-immobilized antibodies.<sup>18,19</sup> The Sips isotherm relates the fractional coverage of antibody binding sites to the concentration of antigen in solution.

$$f = (Kc)^a / [1 + (Kc)^a] \quad (1)$$

Here,  $f$  is the fractional coverage of antibody binding sites and  $c$  is the concentration of unbound analyte. The above expression is based on a model in which the distribution of binding affinities among the immobilized antibodies is described by a Gaussian-like distribution with average affinity  $K$ . The width of this distribution is controlled by the heterogeneity index,  $a$ .<sup>28</sup> If  $a = 1$ , the antigen–antibody binding affinity is described by a single value and the antibodies are homogeneous. Smaller values indicate a wider affinity distribution and thus increasing heterogeneity. In this study, binding isotherms for the Cy5-TNB/anti-TNT Mab interaction were measured for each of the immobilization methods in Figure 2. We then fit the data to eq 1 and compared the resulting  $a$  values to gauge the heterogeneity associated with each immobilization strategy.

**Assessing the Heterogeneity in the Analyte–Antibody Binding Kinetics.** We assessed the heterogeneity in the analyte–antibody association kinetics by collecting kinetic data with the fiber-optic immunosensor and fitting the results to a two-compartment transport–kinetic model.<sup>29,30</sup> This model relates the association and dissociation rate constants for analyte–antibody binding

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as well as the analyte's mass transport properties to the time evolution of analyte binding.

$$\frac{dc_b}{dt} = \frac{k_f}{1 + k_f(c_{b,\text{sat}} - c_b)/k_m} [c(c_{b,\text{sat}} - c_b) - k_r c_b] \quad (2)$$

In the above expression,  $c_b$  is the surface density of bound analyte,  $k_m$  is a phenomenological mass transport coefficient,  $k_f$  is the association rate constant, and  $k_r$  is the dissociation rate constant. Typically, solutions to eq 2 are obtained numerically and fit to experimental data. This approach is now used routinely to obtain estimates for  $k_f$ ,  $k_r$ ,  $k_m$ , and  $c_{b,\text{sat}}$  from biosensor data.<sup>9,29–33</sup>

The two-compartment model describes the association/dissociation kinetics with a simple first-order reaction rate law. It assumes that the antibodies are homogeneous and thus all characterized by the same  $k_f$  and  $k_r$  values. If the kinetics are homogeneous, it should be possible to perform a global analysis by collecting kinetic data at multiple analyte concentrations and fitting all of the data simultaneously to eq 2. If the kinetics are heterogeneous, the kinetic rate constants will change as different subpopulations of antibodies bind analyte. The kinetic parameters will thus be a function of analyte concentration and the sensor will not exhibit unique binding characteristics. Under such circumstances, a global analysis of the data will fail and a comparison between the model and experiment will be poor.

To assess the heterogeneity in the kinetics, we measured the concentration of analyte as a function of time for several different analyte concentrations. For each immobilization strategy, all of these data were fit globally to eq 2. The quality of the fits was then used to judge the presence or absence of heterogeneity in the association/dissociation kinetics.

**Fluorescence Anisotropy Measurements.** To test the validity and accuracy of the Sips isotherm, we measured the binding isotherm for the Cy5-TNB/anti-TNT Mab binding in solution and compared the results with eq 1. The binding isotherm was measured by titrating the antibody with analyte and recording changes in the analyte's fluorescence anisotropy. Experiments were performed in L-format with a PC1 photon counting spectrofluorometer (ISS, Champaign, IL). Samples were excited at 635 nm with a 2-nm band-pass. To maximize sensitivity, a 630-nm interference filter and a 650-nm band-pass filter (Coherent, Auburn, CA) were placed in the excitation and emission light paths, respectively. We titrated a 10 nM antibody solution prepared in PBS with increasing volumes of a 100 nM Cy5-TNB solution in PBS. To ensure that the sample reached equilibrium at each point in the titration, we added analyte and recorded the anisotropy at 5-min intervals until the value was constant within  $\pm 0.005$ . The measured anisotropy was assumed to be a weighted average of the anisotropies of the free and bound Cy5-TNB. The fraction of antibody bound in the sample,  $f_b$ , can thus be related to the

measured anisotropy values.<sup>34</sup>

$$f_b = \frac{r - r_f}{(r_b - r)R + r - r_f} \frac{C_T}{Ab_T} \quad (3)$$

Here,  $R$  is the ratio of the quantum yields for the bound and free analyte,  $r$  is the measured anisotropy,  $r_f$  is the anisotropy of unbound analyte, and  $r_b$  is the anisotropy of bound Cy5-TNB. In addition,  $C_T$  and  $Ab_T$  are the total concentrations of Cy5-TNB and antibody in the sample, respectively. In separate experiments, we determined  $r_f$  by measuring the anisotropy of a 1 nM Cy5-TNB solution in PBS. The same solution was then saturated with a 100-fold excess of the anti-TNT antibody. We measured the anisotropy of this solution to determine  $r_b$ . With estimates for  $r_f$  and  $r_b$ , we determined  $f_b$  from eq 3 for each of the points in the titration and fit the resulting values to the Sips isotherm (eq 1).

**Equilibrium Binding Assays.** Fibers were titrated with 1.5, 3, 5, 7, 8.5, and 10 ng/mL solutions of Cy5-TNB. All solutions were prepared with a running buffer that contained PBS mixed with 2 mg/mL BSA and 0.1% T-20 (PBS/BSA/T-20). To begin the titration, 1.5 ng/mL analyte solution was passed over the fiber surface with a syringe pump at a flow rate of 0.1 mL/min. As the analyte bound, data were collected every 5 min. After the fluorescence signal had equilibrated, the 3 ng/mL analyte solution was introduced over the surface and data were collected in the same way. This procedure was repeated on the same fiber with successively increasing concentrations of analyte. After all of the analyte solutions were injected, the fiber was exposed to a 10 mM solution of Cy5-TNB. In separate experiments, we found that this concentration saturates the majority of the immobilized antibodies on the fiber surface. After the fiber probe was incubated in this solution for 6–8 h at room temperature, the fiber was rinsed with running buffer and the intensity of the fluorescent signal was immediately recorded. All data recorded earlier in the experiment were normalized by this signal so that binding data could be expressed in terms of fractional coverage. Normalizing the data also allowed us to adjust the data for fiber-to-fiber variations.

**Kinetic Assays.** A similar procedure was used to observe the kinetics of Cy5-TNB binding. Binding was observed for four different analyte concentrations: 1.5, 3, 5, and 7 ng/mL. Again, Cy5-TNB solutions were prepared in PBS/BSA/T-20, and binding was observed by injecting the analyte solution over the fiber surface with a syringe pump at a flow rate of 0.1 mL/min. Data were collected every 20 s until the fluorescence signal equilibrated. The fiber surface was then regenerated with a 1:1 mixture of PBS and ethanol. This regeneration step has been shown to have a negligible effect on the activity of the immobilized antibodies. This binding/regeneration procedure was repeated with other analyte concentrations to observe the concentration dependence of the binding kinetics.

## RESULTS

Before assessing the heterogeneity associated with the various immobilization chemistries in Figure 2, we first tested the validity and accuracy of the Sips analysis by characterizing the heterogeneity of the Cy5-TNB/anti-TNT Mab interaction in solution. In

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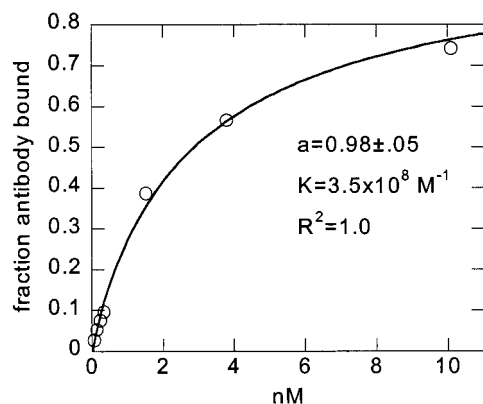


Figure 3. Equilibrium binding isotherm for the interaction between Cy5-TNB and the anti-TNT antibody in solution. A least-squares fit to the Sips isotherm (solid line) is also shown along with estimates for the heterogeneity index,  $a$ , and the average antibody binding affinity,  $K$ .

these experiments, the binding affinity should be homogeneous ( $a = 1$ ) since (1) the antibodies are monoclonal and (2) steric effects that can introduce heterogeneity into the binding affinity of immobilized antibodies are not relevant to soluble proteins. The equilibrium binding isotherm was determined from fluorescence polarization/anisotropy measurements. The results of these experiments are presented in Figure 3 along with least-squares fits to eq 1. The Sips isotherm clearly provides a good description of binding. As expected, the Cy5-TNB/anti-TNT Mab interaction displays a homogeneous binding affinity since the heterogeneity index is nearly unity. Therefore, the Sips isotherm appears to be a reasonable method of quantifying the heterogeneity in the analyte–antibody binding affinity.

Figure 4 shows the measured isotherms for the binding of soluble Cy5-TNB to immobilized antibodies. Nonlinear least-squares fits with the Sips isotherm appear with the experimental data. Like the measurements in solution, the Sips isotherm provides an excellent description of analyte–antibody binding. Figure 4 also gives the heterogeneity indexes and binding affinities for each of the immobilization schemes. Most of the immobilization strategies yielded heterogeneity indices less than 1. Affinities fell between  $6.1 \times 10^6$  and  $7.0 \times 10^7 \text{ M}^{-1}$ . These values are  $\sim 1$  order of magnitude smaller than the corresponding affinities in solution.

In a previous investigation, Zeck et. al. measured affinity constants for the binding of several TNT analogues to the A1.1.1 anti-TNT antibody immobilized on polystyrene plates.<sup>35</sup> The values in Figure 4 are  $\sim 1$  order of magnitude smaller than their published values. None of the TNT analogues used in the earlier study contained bulky fluorescent tags, and it is possible that the steric impediment due to the analyte's Cy5 tag reduced the binding affinity in our studies. In any case, the lower affinity constants and increased heterogeneity following surface coupling clearly indicate that immobilization introduces heterogeneity into the analyte–antibody binding behavior and diminishes antibody activity.

It should be noted that the data in Figure 4 were collected at relatively low analyte concentrations, which are typical of the

operating conditions in many environmental TNT assays.<sup>1,23,36</sup> This choice of concentrations permitted us to study analyte binding under the conditions used in practice. However, it was too narrow to capture the entire analyte–antibody binding isotherm since only 10–40% of the available antigen binding sites are observed in Figure 4. Our analysis, therefore, strictly does not assess the heterogeneity in the entire antibody population. Selinger and Rabbany showed that analyzing binding isotherms with the Sips approach at such low concentrations only yields information about the shape of the high-affinity tail of the assumed Gaussian-like affinity distribution.<sup>19</sup> In the low concentration limit, the heterogeneity index describes the rate at which this tail decays. When  $a \geq 1$ , the tail decays rapidly and the measured isotherm appears homogeneous. In contrast, smaller  $a$  values correspond to a slowly decaying tail and therefore increasing heterogeneity. Thus, rather than characterizing the entire protein population, the results in Figure 4 only truly describe the heterogeneity of the antibodies with the highest affinities.

Nevertheless, these data may still provide information about the heterogeneity in the entire antibody population. If the Gaussian-like distribution that is assumed in the Sips model is realistic, the heterogeneity in the tail of the distribution should be directly correlated with the heterogeneity of all the immobilized proteins. To test this hypothesis, we measured the complete binding isotherm for the randomly oriented and the direct carbohydrate attachment chemistries depicted in Figure 2. Again, the data are well described by the Sips isotherm (Figure 5). More importantly, the affinity and heterogeneity indexes agree with the corresponding values measured at lower analyte concentrations. Thus, although we have not examined all of the chemistries in Figure 2, our results indicate that the binding data obtained at low concentrations reflect the heterogeneity and affinities of the entire antibody population.

Although most of the immobilization strategies in Figure 2 yielded heterogeneous populations of antibodies, some of the immobilization schemes did produce more uniformly reactive surfaces. In particular, linking antibodies either directly to the surface through the Fc region's carbohydrate moiety or indirectly through an oriented streptavidin layer generated surfaces with heterogeneity indexes between 0.9 and 1. As one would intuitively predict, these strategies uniformly orient the proteins by site-selective attachment to a uniform support. For example, with randomly immobilized streptavidin, the heterogeneity indexes were still relatively small ( $a \sim 0.7\text{--}0.8$ ). However, the heterogeneity index increased to  $a = 1$  when the streptavidin was oriented on the fiber surface. The Sips isotherm, therefore, provides a quantitative evaluation of the relative heterogeneity achieved with these approaches.

Although some of our observations suggest that the extent of heterogeneity is largely controlled by antibody orientation, other factors could also explain the variation in the apparent heterogeneity. For example, the variability in the surface chemical microenvironment or the extent of protein denaturation may differ among the tested immobilization strategies. Apparent variations in the antibody heterogeneity may also arise if the analyte adsorbs nonspecifically to the fiber surface rather than to the antibody binding sites. Finally, contributions to the fluorescence signal from

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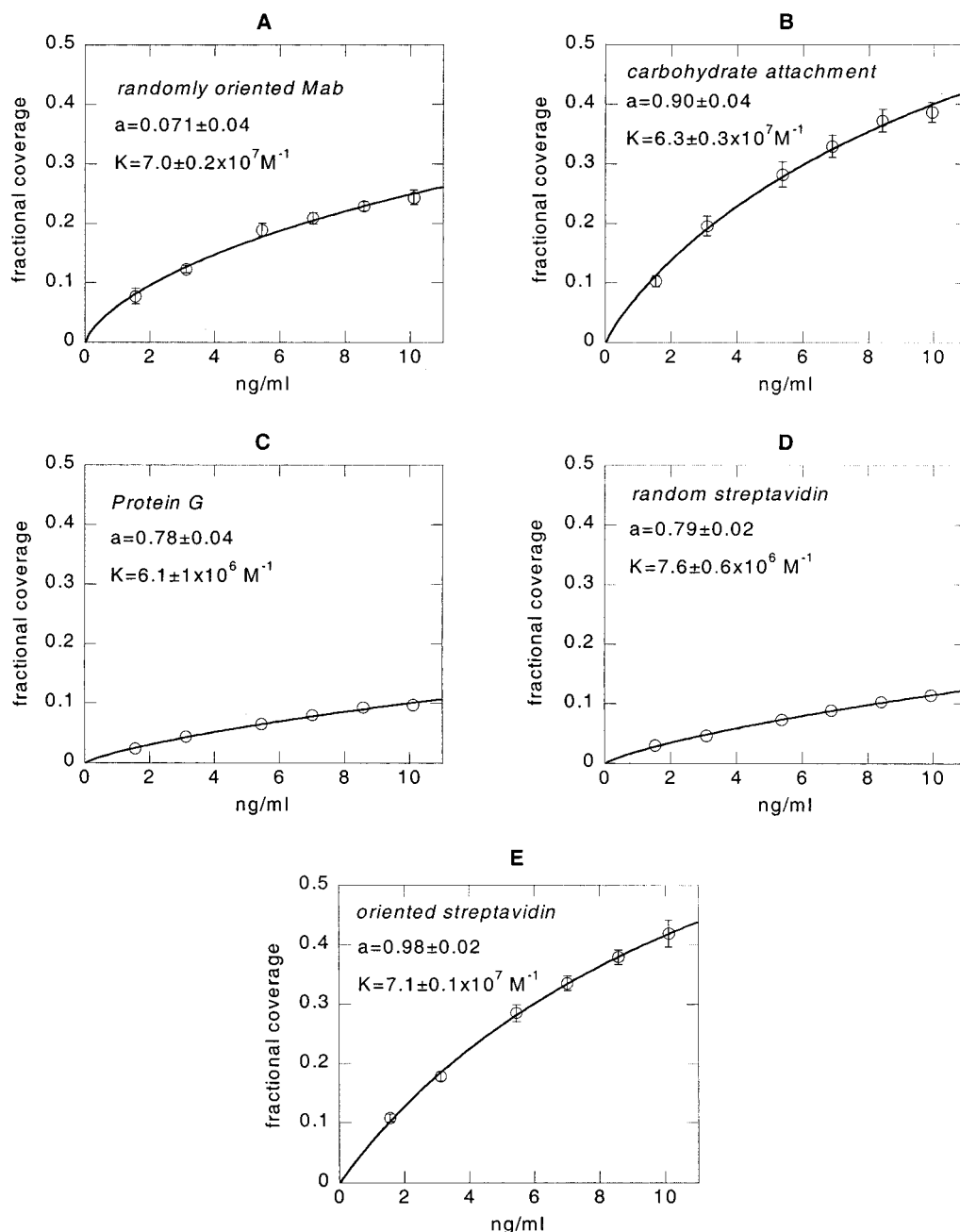


Figure 4. Equilibrium binding isotherms for the adsorption of Cy5-TNB onto surface-immobilized anti-TNT antibodies. Experimental data (open circles) were fit to the Sips isotherm (solid lines) for each of the immobilization chemistries in Figure 2. In all cases, the fits yielded  $R^2$  values greater than 0.95. Values for the heterogeneity index,  $a$ , and average antibody affinity,  $K$ , from these fits are also listed for each immobilization chemistry. Experimental data points represent the mean  $\pm$  SEM of data collected with three different fiber-optic probes.

bulk, unbound analyte near the fiber surface can also distort our results.

To investigate the importance of nonspecific adsorption and signals from the bulk sample solution, we studied the interaction between Cy5-TNB and surface-immobilized goat IgG. Goat IgG was coupled to fiber-optic probes via each of the described immobilization strategies. In all cases, the measured fluorescent signals were negligible compared to the corresponding signals collected with the anti-TNT antibody (data not shown). Assuming that the nonspecific adsorption characteristics of Cy5-TNB are the same on goat IgG and the anti-TNT antibody, this result shows that the contributions from nonspecific adsorption and bulk analyte

are negligible compared to the signal that arises from the specific binding of the Cy5-TNB to the anti-TNT antibody.

It should be noted that the heterogeneity indexes and binding affinities reported in Figure 4 may actually overestimate their true values. These quantities are determined from the fractional coverages that also appear in Figure 4. Although we saturated the fibers with analyte, the fractional coverage does not account for inactive protein. Accounting for inactive antibodies would result in smaller, measured fractional coverages and higher antibody heterogeneity. Thus, given a sufficiently high density of inactive protein, the true  $a$  and  $K$  values would be smaller than the values in Figure 4 so that the loss of activity and heterogeneity of the



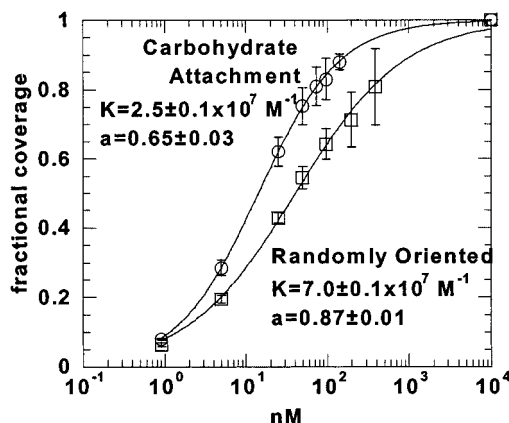


Figure 5. Complete equilibrium binding isotherms for the binding of Cy5-TNB onto fiber-optic probes prepared with the randomly oriented (squares) and the carbohydrate attachment (circles) chemistries illustrated in Figure 2. Solid lines represent best fits to the Sips isotherm. For both immobilization chemistries, the fits yielded  $R^2$  values greater than 0.99. Values for the heterogeneity index,  $a$ , and average antibody affinity,  $K$ , from these fits are also listed. Experimental data points represent the mean  $\pm$  SEM of data collected with three different fiber-optic probes.

immobilized proteins would be even more pronounced than our observations suggest.

Ideally, it is desirable to use an immobilization strategy that maximizes the activity of all the immobilized antibodies and thereby minimizes the amount of inactive protein. In light of this, it is important to consider both active and inactive forms of the antibody when the biological activities associated with each of the tested immobilization chemistries are evaluated. We therefore did not compare the affinities in Figure 4 directly, but instead measured the specific binding capacity (mass of bound analyte/mass of immobilized protein). This quantity was determined from the ratio of the maximum analyte surface coverage at saturation to that of the total antibody.<sup>4,15</sup> Here, we performed radiolabeling measurements to determine the total density of antibody on the fiber surface and used the maximum fluorescence signal at saturation as a measure of analyte density on the fiber probes. This approach should be accurate since nonspecific binding and the bulk analyte solution do not contribute to the fluorescence signal. Unlike the binding affinities determined from the Sips isotherm, the specific binding capacity accounts for all of the antibodies on the fiber surface and should be a more accurate measure of biological activity.

Values for the antibody surface density and specific activity are listed in Table 1. There is no direct relationship between the extent of heterogeneity and the specific activity of the immobilized antibodies. Our results show that chemistries that yield more homogeneous, active protein populations do not guarantee higher specific binding capacities and more active surfaces. For example, antibodies immobilized on oriented streptavidin layers produced the most homogeneous binding ( $a \sim 1$ ). However, the specific activity is still comparable to that of their randomly oriented counterparts. This suggests that a relatively large fraction of the antibodies were inactivated by this immobilization strategy. At the other extreme, antibodies coupled to protein G or randomly oriented streptavidin layers exhibited heterogeneous binding affinities ( $a \sim 0.7$ – $0.8$ ), but they nevertheless displayed specific

Table 1. Comparison of Antibody Surface Density and Immunological Activity for the Immobilization Chemistries in Figure 2

immobilization chemistry	antibody surface density (ng/mm <sup>2</sup> )	specific activity (ng/mm <sup>2</sup> ) <sup>-1</sup>
randomly oriented antibody	$1.7 \pm 0.2$	$4100 \pm 800$
direct carbohydrate	$0.84 \pm 0.17$	$11400 \pm 3600$
protein G	$0.72 \pm 0.12$	$9900 \pm 2900$
random streptavidin	$0.67 \pm 0.05$	$1800 \pm 900$
oriented streptavidin	$0.42 \pm 0.14$	$3800 \pm 1400$

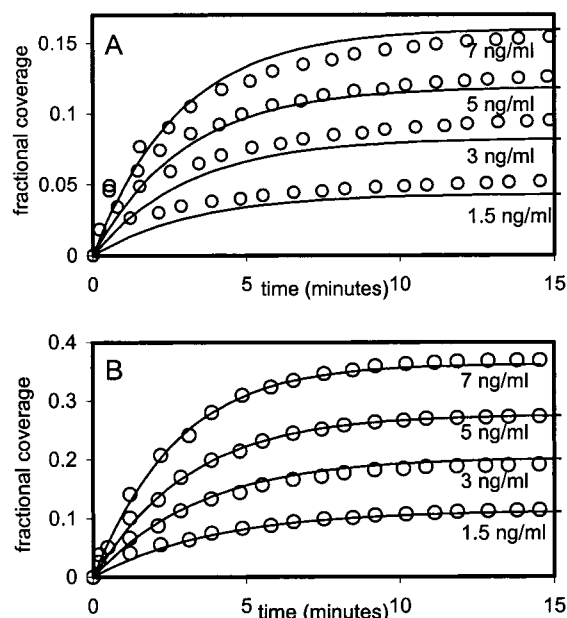


Figure 6. Kinetic data for antibodies (A) randomly oriented on the fiber surface and (B) directly attached via their carbohydrate regions fit globally to a two-compartment transport-kinetic model (solid lines).

binding capacities that were only slightly smaller than that of the randomly oriented protein. In these cases, the coupling schemes may have preserved the activity of a larger fraction of the antibodies. For other immobilization chemistries, however, higher biological activity did accompany more homogeneous binding. In particular, directly attaching antibodies to the surface through their carbohydrate regions produced surfaces that exhibited high specific binding capacities and uniform reactivity.

In the final part of this study, we assessed the heterogeneity in the kinetic rate constants for Cy5-TNB/anti-TNT Mab binding by globally analyzing data obtained at different analyte concentrations. To do this, we focused the interaction between the analyte and antibodies, which were directly linked to the fiber surface via their carbohydrate moieties. We also compared kinetic data for randomly oriented antibodies. Kinetic data for both of these immobilization chemistries are presented in Figure 6. Global analyses of these data are also shown.

For both immobilization chemistries, it was possible to globally fit the kinetic data to the two-compartment model. Estimates for the model's parameters are given in Table 2. In both cases, the values for the fitted parameters are reasonable. The ratios of the association and dissociation rate constants are in good agreement with the previously reported affinities. Nevertheless, a qualitatively better fit was obtained when the antibodies were immobilized

Table 2. Rate Constants, Antibody Density, and Mass-Transfer Coefficients Estimated by Globally Fitting the Data in Figure 6 to the Two-Compartment Transport–Kinetic Model<sup>a</sup>

	$k_f$ ( $10^5$ $M^{-1}s^{-1}$ )	$k_r$ ( $10^{-3}$ $s^{-1}$ )	$C_{b,sat}$ ( $10^{-8}$ $mol/m^2$ )	$k_m$ ( $10^{-6}$ $m/s$ )
randomly oriented antibody	$4.3 \pm 0.07$	$5.7 \pm 0.1$	$1.1 \pm 0.3$	$9.0 \pm 0.3$
direct carbohydrate attachment	$1.5 \pm 0.1$	$6.0 \pm 0.4$	$1.5 \pm 0.1$	$10 \pm 1$

<sup>a</sup> Error estimates are calculated from each parameter's standard error.

through the carbohydrate on the Fc domain. By contrast, with the randomly oriented antibodies, the global fit either overestimates or underestimates the binding kinetics, depending on the analyte concentration and the extent of binding on the surface. This suggests that the kinetic rate constants for randomly bound antibody are not unique but exhibit a distribution of values. In contrast, when the antibodies are coupled to the fiber surface via their carbohydrate regions, the better fit suggests that the kinetic rate constants are described by a single set of values and are thus homogeneous.

## DISCUSSION

In summary, this study characterized the effect of several antibody immobilization strategies on the heterogeneity of the bound protein. We applied the Sips isotherm to quantify the heterogeneity in the analyte–antibody binding affinity. Antibodies that were randomly immobilized displayed heterogeneous binding affinities. However, other immobilization strategies that tend to uniformly align the antibodies on the supporting surface yielded more homogeneous behavior. In particular, our results indicate that immobilizing antibodies through their carbohydrate moiety is the most promising surface attachment strategy among those tested. With this method, we observed the highest biological activity and the most homogeneous binding affinities. These antibodies also exhibited more homogeneous binding kinetics than antibodies that were randomly oriented on the supporting surface. These results suggest that the extent of heterogeneity in the antibody affinity is directly related to the degree of heterogeneity in the analyte–antibody kinetics. This is not surprising since the same physicochemical mechanisms (i.e., steric effects, chemical microenvironment, etc.) that lead to heterogeneity in binding affinity are probably responsible for variability in the kinetic parameters as well.

The accuracy of our heterogeneity analysis depends on the assumed Gaussian-like distribution of affinities that underlies the Sips isotherm. It is possible that other isotherms provide a better description of binding. In particular, rather than being distributed continuously, the affinity may be concentrated at discrete values. To investigate this possibility and also test the assumptions behind the Sips isotherm, we considered several models in which the affinity is distributed among multiple states or values. In these models, each state can be described by a Langmuir isotherm. The complete isotherm is thus given by the following expression.

$$f = \sum_{i=1}^N \frac{x_i K_i c}{1 + K_i c} \quad (4)$$

Here,  $N$  is the total number of states.  $K_i$  and  $x_i$  are the affinity and mole fraction of state  $i$ , respectively. We attempted to fit the data in Figures 4 and 5 to eq 4 with  $N = 2$  and  $N = 3$ . Although the data fit qualitatively, the uncertainties in the affinities and mole fractions were unreasonably large (data not shown). We thus concluded that such discrete affinity distributions do not accurately describe analyte–antibody binding. The continuous distribution of affinities described in the Sips model still provides a much better description of binding.

Besides providing information for the construction of improved protein-based devices, our results are also useful to researchers who use biosensors to investigate the kinetic and thermodynamic properties of biomolecular interactions.<sup>29,31,37</sup> In previous studies, investigators also globally analyzed kinetic data to measure rate constants. However, as we have shown in this and previous work, this approach is only successful if the binding kinetics of the immobilized protein population are homogeneous.<sup>9</sup> In the case of antibody-based sensors, our results (Figure 6) clearly show that immobilizing antibodies through their carbohydrate component satisfies this requirement.

One should note that we did not optimize the procedures that were used to immobilize antibodies on the fiber-optic probes. It may be possible to further increase antibody activity and achieve more homogeneous binding by modifying the immobilization conditions (i.e., antibody and reagent concentrations, incubation times, etc.). Varying the immobilization procedures would most likely alter protein activity and heterogeneity by modifying the density of antibodies on the fiber-optic probes. Several studies demonstrated that increased antibody density can augment surface activity. However, these same studies also showed that, beyond an optimum surface density, the apparent antibody activity decreases due to steric hindrance between neighboring antibodies.<sup>3,4</sup> In addition, it is possible that the extent of antibody heterogeneity can also change with antibody surface coverage. At sufficiently high surface densities, immobilized antibodies can form two-dimensional clusters.<sup>38</sup> In these clusters, the lateral protein–protein interactions may order the antibodies so that they exhibit more uniform binding.

Besides antibody surface density, other variables may also impact protein activity and heterogeneity. In particular, the binding behavior may depend on the physical characteristics of the solid support such as the roughness of the underlying matrix. The irregular surface topology of rough substrates could randomize the orientation of the attached antibodies so that analyte–antibody binding becomes more heterogeneous. In addition, the size of the analyte may play a pivotal role. Cy5-TNB is a relatively small molecule when compared to the size of the immobilized antibodies. Clearly, steric effects and therefore antibody heterogeneity will be more pronounced if a much larger analyte is used. Additional experiments are underway to study antibody heterogeneity with different antibody surface densities, various solid supports, and larger analyte molecules.

Finally, we have only investigated a single analyte–antibody system. Although we have not assessed the heterogeneity of other antigen–antibody or receptor–ligand interactions, the results of this study could be useful for characterizing other immobilized

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proteins. In particular, this study demonstrates that the Sips isotherm is a promising method for assessing heterogeneity. It provides a good description of analyte binding for both surface-bound and soluble proteins. Moreover, the Sips isotherm correctly reflects the extent of heterogeneity. It properly indicates (1) homogeneous binding in solution and (2) more homogeneous binding for more uniformly oriented antibody films. The Sips isotherm may thus be a useful tool for assessing heterogeneity and selecting immobilization strategies for applications that use immobilized proteins.

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