

Research Article

Characterization for Binding Complex Formation with Site-Directly Immobilized Antibodies Enhancing Detection Capability of Cardiac Troponin I

Il-Hoon Cho,¹ Sung-Min Seo,¹ Jin-Woo Jeon,¹ and Se-Hwan Paek^{1,2}

¹Program for Bio-Microsystem Technology, Korea University, 1, 5-ka, Anam-dong, Sungbuk-ku, Seoul 136-701, South Korea

²Department of Biotechnology and Bioinformatics, Korea University, Jochiwon, Choongnam 339-800, South Korea

Correspondence should be addressed to Se-Hwan Paek, shpaek@korea.ac.kr

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The enhanced analytical performances of immunoassays that employed site-directly immobilized antibodies as the capture binders have been functionally characterized in terms of antigen-antibody complex formation on solid surfaces. Three antibody species specific to cardiac troponin I, immunoglobulin G (IgG), Fab, and F(ab')₂ were site-directly biotinylated within the hinge region and then immobilized via a streptavidin-biotin linkage. The new binders were more efficient capture antibodies in the immunoassays compared to randomly bound IgG, particularly, in the low antibody density range. The observed improvements could have resulted from controlled molecular orientation and also from flexibility, offering conditions suitable for binding complex formations.

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1. Introduction

Immuno-analytical systems have typically been developed by capturing and immobilizing the antibody on solid surfaces since the immobilization provides an easy separation of the binding complexes with the antibody from unreacted reagents [1]. The solid-phase capture antibody, however, is very difficult to control at the level of the molecular state or orientation on the surfaces, which is a major factor that determines the degree of the complex formation [2]. Indeed, most immobilization methods (e.g., physical adsorption, covalent attachment, and binding via biotin-streptavidin linkage) [2, 3] have not yet been very successful in arranging or orientating the antibody molecules [4] in a manner that substantially improves the random nature of antibody immobilization inherent in the current methods. Random immobilization results in a low rate (e.g., 5 to 10%) of the active antibody density, that is, those that can participate in the binding reaction, which decreases even further when the substance to be analyzed is large in molecular size [2].

The rate of active antibody density would significantly increase provided the molecules are deposited by mediating the Fc region on the solid matrix, such that the antibody

binding sites may face to the bulk solution where the analyte is present. As a typical example, protein A or G binds specifically to the Fc region of immunoglobulin [5] and, thus, the capture antibody can be immobilized via this binding protein in the “bottom-on” configuration. It has been demonstrated that such a controlled molecular orientation of the antibody significantly improves the binding rate compared to random immobilization of the antibody by up to about 10 times for the same antigen [3]. The functional binding affinity measured for the oriented antibody could also increase because the molecules are properly posed to orient the binding sites at a position distal from the surfaces. Such improved characteristics would consequently enhance the analytical performances of solid-phase immunoassays [3, 6]. However, it is worth noting that the use of antibodies immobilized via the binding proteins is limited to only competitive-type assays in which only a single antibody is used.

To develop an immobilization scheme that can be used in a wide range of applications, we have explored site-directed biotinylation of antibodies to bind the molecules via a biotin-streptavidin linkage on the solid surfaces [7]. Since the hinge region of immunoglobulin G (IgG) could be a potential site

for coupling to the matrix, a novel method was devised to selectively attach a biotin derivative to the functional groups present within this region. Although this product, if used as the capture antibody, led to an enhancement in the performance of the sandwich-type immunoassay, the Fc region in the molecule was likely interfere with immobilizing the antibody in an erect position, which is desirable for binding. To address this, we next synthesized a product biotinylated at the same region but using an antibody fragment, F(ab')₂, in place of IgG [3]. The use of this capture antibody further enhanced the performances of the immunoassay, indicating that the molecules could not only be positioned on the streptavidin layer in an arrangement that efficiently exposed the binding sites to the bulk solution, but also with a high flexibility for access to the antigen. Such improvements would be crucial to the analytical systems for, particularly, cardiac troponin I (cTnI), as a specific marker of acute myocardial infarction (AMI), requiring a high detection capability.

In this study, we analyzed the complex formation with the immobilized antibodies to cTnI prepared in different schemes using variable biotinylation methods and antibody types. In the previous report, the site-directly biotinylated antibodies were shown to be superior to the conventional random preparation if they were used as the capture antibodies in sandwich immunoassays [3]. As to the molecular types, since the fragmented antibody without the Fc had an enhancement effect, the smaller fragment, Fab, was also employed in this study to examine a new factor, that is, the number of binding units per molecule. The respective complex formation with such different preparations have been analyzed with respect to two major parameters determining the reaction; the surface density of active binding sites and binding affinity. Based on these analyses, we have attempted to construct binding models that represent the reactions of each antibody with the antigen on the solid surfaces. The models could consequently be used to elucidate the effects of the following factors on the complex formation: biotinylation method, the presence of Fc, and the number of binding units per molecule.

2. Materials and Methods

2.1. Materials. The stock of cTnI-T-C complex (from human cardiac muscle tissue) and mouse monoclonal antibody specific to cTnI (Clone 19C7) were supplied by HyTest (Turku, Finland). Goat anti-mouse IgG antibody, two types of goat anti-mouse IgG antibodies (specific to whole IgG and Fc of mouse IgG, resp.) conjugated with horseradish peroxidase (HRP), succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (SMCC), 1-biotin-amido-4-(4'-[maleimidoethyl]cyclohexane)-carbox-amido)butane (biotin-BMCC), succinimidyl-6-[biotin-amido]-6-hexanamidohexanoate (NHS-LC-LC-biotin), and ImmunoPure IgG₁ Fab and F(ab')₂ preparation kit were purchased from Pierce (Rockford, IL). Mercaptoethanol (ME), dithiothreitol (DTT), sephadex G-15 gel, 3,3',5,5'-tetramethylbenzidine (TMB), casein (sodium salt type, extracted from milk), glutaraldehyde (GA), protein marker

(low range), and polyoxyethylene sorbitan monolaurate (Tween 20) were obtained from Sigma (St. Louis, MO). Streptavidin and HRP were obtained from Calbiochem (San Diego, CA). Ficin (from a fig tree) protease was purchased from Wako (Osaka, Japan). A protein G affinity column was supplied by Bio-rad (Hercules, CA). Other reagents used in this study were analytical grade.

2.2. Substrate Solution for HRP. The substrate solution for the microtiter-plate-based immunoassays contained 10 mL of 50 mM sodium acetate (pH 5.1), 100 μ L of 1% TMB, and 10 μ L of 3% hydrogen peroxide.

2.3. Preparation of Antibody Fragments

2.3.1. Production of Fab. A monoclonal antibody specific to cTnI (BD clone 12) was produced by following a standard protocol as described elsewhere [8]. The antibody was fragmented using the ImmunoPure IgG₁ Fab and F(ab')₂ Preparation Kit according to the manufacturer's instructions. Briefly, the Ficin column (gel volume: 2 mL) was equilibrated with activation buffer containing 10 mM cysteine to eventually yield Fab as the product. The antibody solution, diluted to 1 mg/mL in phosphate buffered saline (PBS), was added to the equilibrated Ficin column, and incubated at 37°C for 5 hours. The digested antibody solution eluted from the column was immediately transferred to the protein A column (gel volume: 2.5 mL). After collection of the unbound fractions, the bound antibody components were collected with the elution buffer supplied in the kit.

Each fraction obtained from the protein A column was then analyzed by means of a solid-phase immunoassay with an immobilized anti-mouse IgG antibody. The goat anti-mouse IgG antibody (1 μ g/mL, 100 μ L) dissolved in PBS was added to microwells and incubated within a container maintained at 100% relative humidity and 37°C for 1 hour. These incubation conditions were also applied to all subsequent reaction steps. After washing the microwells three times with deionized water, the residual surfaces were treated with 100 mM Tris buffer, pH 7.6, containing 0.5% casein (Casein-Tris; 200 μ L). After washing, each eluted fraction from the protein A column were diluted 100 times with Casein-Tris containing 0.1% Tween (Casein-Tris-TW) and added (100 μ L) to duplicate wells. After washing, two types of anti-mouse IgG antibodies (specific to whole IgG and Fc region of mouse antibody, resp.), which were labeled with HRP, were diluted 5000 times with Casein-Tris-TW and subsequently added (100 μ L) to each sample. After washing, the substrate solution for HRP (200 μ L) containing soluble TMB was dispensed into each well and reacted at room temperature for 15 minutes. Sulfuric acid (2 M, 50 μ L) was added, and the absorbance was then measured at 450 nm with a microtiter plate reader (VERSAmix, Molecular Device, Chicago, IL).

The eluted fractions were also characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (12% gel) under non-reducing conditions, as described elsewhere [3].

2.3.2. Production of $F(ab')_2$. The same monoclonal antibody described in the production of Fab section was also fragmented by Ficin protease dissolved in digestion buffer (50 mM Tris-HCl containing 2 mM EDTA, pH 7.0). The antibody (1 mg) was mixed with the dissolved Ficin solution (molar ratio of Ficin to antibody was 1 to 30) containing 1 mM cysteine as an activator and the mixture was then incubated at 37°C for 2 hours. After the enzymatic digestion, the mixed solution was immediately transferred to the protein G column (gel volume: 1 mL), which had been pre-equilibrated with 10 mM PBS (pH 7.4). After collecting the unbound fractions, the bound components were eluted with 0.2 M glycine buffer (pH 3.0). Each fraction from the column was then analyzed by of the Bradford assay at 595 nm [9].

To characterize the unbound and bound fractions, SDS-PAGE and solid-phase immunoassay were employed, respectively. The conditions used for the SDS-PAGE analysis (12% gel) are described elsewhere [3]. In the immunoassay, the goat anti-mouse IgG antibody (1 $\mu\text{g}/\text{mL}$, 100 μL) dissolved in PBS was added to microwells and incubated within a container maintained at 100% relative humidity and 37°C for 1 hour. In this immunoassay, the incubation and washing procedure of each reaction step were the same as the conditions described in the production of Fab section. After blocking the residual surface with Casein-Tris, each unbound fraction, bound fraction and $F(ab')_2$ fragment, pre-made with a commercial kit as a control, was serially diluted (0 to 1000 ng/mL) with Casein-Tris-TW and then added to microwells. Following the reaction, anti-mouse IgG labeled with HRP was added and signal generation was performed by the same procedures described above.

2.4. Synthesis of Labeled Antibodies

2.4.1. Labeling Antibody with Biotin. The antibody molecule can be biotinylated with a few commercially available cross-linkers, which are reactive to some chemical functions that exist on the outer surface of the antibody. We prepared three types of antibody species (i.e., IgG, $F(ab')_2$ and Fab) and then produced the biotinylated antibodies using not only an amine-reactive biotin (NHS-LC-LC-Biotin) linker for random biotinylation of IgG type but also a sulfhydryl-reactive biotin (biotin-BMCC) linker for site-directed biotinylation of IgG, $F(ab')_2$ and Fab types in this study. For random biotinylation of IgG and site-directed biotinylation of IgG and $F(ab')_2$, the components were prepared according to protocols we previously published [3]. In addition, unlike the two antibodies from the site-directed biotinylation method, that is, IgG and $F(ab')_2$, synthesis of site-directed biotinylation of Fab was performed by a simple biotinylation process (five-fold molar excess of biotin-BMCC) without inducing the competitive reaction among maleimide derivatives [3] because Fab fragment molecule contains just one disulfide bond that can be reduced by reducing agents at the interchain between C_{H1} and C_L domain. After site-directed biotinylation of Fab, excess biotin-BMCC reagent was removed on a Sephadex

G-15 gel column (gel volume: 10 mL). The biotinylated Fab was then concentrated and stored at 4°C until use.

2.4.2. Labeling Antibody with HRP. The monoclonal antibody, clone 19C7, was coupled to the enzyme, that is, HRP, via a cross-linking protocol that was similar to a protocol previously described elsewhere [10]. Briefly, the antibody was first reduced using 10 mM DTT at 37°C for 1 hour, and the excess reagent was removed on Sephadex G-15 gel column (10 mL volume). HRP was also activated with SMCC in a 50 molar excess, and the excess reagents were also separated on the Sephadex G-15 gel column. The reduced antibody was then combined with the activated HRP in a 5 molar excess and the conjugation was carried out overnight at 4°C. The conjugates synthesized were stored as aliquots after snap freezing.

2.5. Performance Characterization of Immobilized Antibodies

2.5.1. Analytical Procedure. To analyze the performance of the four different biotinylated antibodies, that is, random IgG, site-directed IgG, site-directed $F(ab')_2$, and site-directed Fab, as capture agents, antibody immobilization on the inner surfaces of microwells were performed by a streptavidin-biotin linkage [11, 12]. A streptavidin solution (10 $\mu\text{g}/\text{mL}$, 100 μL) dissolved in PBS was added to the microwells pre-treated with 0.5% glutaraldehyde and reacted within a humidified container at 37°C for 1 hour. These incubation conditions were used in all subsequent steps, and the wells were washed with deionized water three times after completing each step. After treating the well surfaces with Casein-Tris, the four types of biotinylated antibodies dissolved in Casein-Tris-TW were serially diluted (0.5, 5, 25, and 75 pmol/mL) and added to each microwell. Antigen-antibody complexes were then formed by sequentially adding variable analyte concentrations of cTnI-T-C (0, 0.01, 0.1, 1, 10, 100, 1000, and 2000 ng/mL), and a constant amount of mouse anti-cTnI antibody-HRP conjugates (1 $\mu\text{g}/\text{mL}$). The rest procedure for signal generation and measurement were performed by the same procedures described above.

2.5.2. Data Manipulation. To investigate the antigen binding patterns of the four types of biotinylated antibody participants immobilized on streptavidin layer, we analyzed the data obtained from the two-site immunoassay as described above. The colorimetric signal data obtained from the each analyte concentration were quantified as molar concentration using a linearized HRP standard curve [2]. The numerical values were then transformed into the analytical parameters for Scatchard plot analysis [13]. The surface density of the reactive binding site and the equilibrium binding constant were determined from the x-intercept and slope of the regression curve.

3. Results and Discussion

To improve detection ability of cTnI, immunoassay systems employing different capture antibodies were investigated in this study by immobilizing them, via a biotin-streptavidin

linkage, in various configurations [3]. It has been reported that the adoption of the site-directly biotinylated antibody was superior in its analytical performance over the use of the randomly prepared biotinylated antibody. Furthermore, the presence of the Fc region in the site-directly biotinylated antibody molecule may result in a steric effect that was significantly alleviated provided an antibody fragment, $F(ab')_2$, was employed.

The aim of this study was to continue this work by characterizing not only the surface density of immobilized captured antibodies that were actively participating in the reaction but also the binding affinity, which primarily determines analytical performance. It is worth noting that the Fab fragment, which was used as the capture antibody and could determine the minimal size of the binder, was newly synthesized to more fully determine the controlling factors.

3.1. Preparation of Antibody Fragments

3.1.1. Production of Fab. The smaller antibody fragment was produced by enzymatically digesting the IgG₁ mouse antibody using a protease, Ficin, immobilized on a gel column. To derive the reaction for mainly synthesizing Fab, the antibody was incubated with 10 mM cysteine, which acted as an activator, within the column for an extended time period (e.g., 5 hours) [14]. After collecting the reaction mixture from the Ficin column, the product was sequentially purified by affinity chromatography on a protein A column. The unbound portion was retrieved as fractions and the bound portion was eluted by shifting the medium to a low pH buffer. Each fraction was analyzed for total antibody with a sandwich-type immunoassay using a secondary antibody, as the capture binder, raised against mouse whole IgG and the same antibody but chemically conjugated to an enzyme, HRP, as the detection molecule. To monitor Fab, the same immunoassay system was employed except with the use of a detection antibody specific only to the Fc region. The immunoassay results, as shown in the chromatogram in Figure 1(a), revealed that the major component in the unbound fractions in the first peak was Fab and the components in the bound fractions in the second peak contained Fc, including the non-digested IgG.

To verify that the major component in the unbound fraction was Fab, the fractions were pooled and analyzed by means of SDS-PAGE under non-reducing conditions (Figure 1(b)). This analysis showed that the major protein band had a molecular weight between 66 and 45 kDa. Impurities were also present although they were relatively minor. Since the molecular size of Fab is approximately 50 kDa, we can conclude that this major protein band is Fab cleaved from IgG by Ficin. It should be noted that the yield of Fab fragmentation by enzymes was approximately 50% provided the areas under each antibody fragment peak were compared with each other (refer to the curve marked with open square in Figure 1(a)).

3.1.2. Production of $F(ab')_2$ in Large Scale. Unlike the above enzymatic digestion in the solid phase, $F(ab')_2$ was

synthesized by carrying out the same cleavage reaction in the solution phase containing 1 mM cysteine as an activator. This method provided an improved yield of the reaction when compared to the previous method although the enzyme could not be reused. The antibody fragments were then purified by affinity chromatography on a protein G column, which was essential for separating $F(ab')_2$ in a high purity from the reaction mixture containing the enzyme. After eluting the unbound fractions, the bound material was dissociated from the column by switching the medium to a low pH buffer. To obtain the chromatogram the protein concentration in each elution fraction was analyzed by the Bradford assay (Figure 2(a)).

After pooling the fractions in each peak, the components were analyzed by SDS-PAGE under non-reducing conditions (the inset, Figure 2(a)). This analysis showed that the unbound fractions included mainly the smaller fragment, Fc, which had a molecular weight of about 50 kDa, and the bound fraction contained the larger fraction, $F(ab')_2$, which had a molecular weight of about 100 kDa. These results indicate that this method resulted in a complete isolation of the product. The gel data, however, was in conflict with the preconception that protein G specifically binds to the Fc region of the antibody molecule, which would have resulted in the opposite order of the eluted components. To settle this contradiction, each fragment was reacted with the antigen, cTnI, immobilized on the solid matrix, and the complex was traced using a secondary antibody, raised against mouse IgG, conjugated to an enzyme, HRP (Figure 2(b)). The fragment that came from the bound fractions showed a binding pattern to the antigen similarly to that obtained with the $F(ab')_2$ control. These results were supported by a previous study [15] where $F(ab')_2$ produced by the enzymatic digestion of the same subtype antibody using Ficin was found to strongly bind to protein G whereas the cleaved Fc poorly reacted with the binding protein under physiological conditions.

The liquid phase cleavage reaction had several advantages relative to the solid phase cleavage employed in the earlier investigation [3]. First, as shown in the SDS-PAGE data, only the two Fc and $F(ab')_2$ components were contained in the unbound and bound peaks, respectively. This indicated that the yield of fragmentation under the conditions used was close to 100%, which was a significant improvement over the solid phase reaction (about 20 to 30%). Secondly, the antibody cleavage was completed via enzymatic digestion within 2 hours, which was about one-tenth the reaction time required for the previous protocol (approximately 20 hours). Finally, the reproducibility of the cleavage reaction was remarkable as shown in the quadruplicate chromatograms of Figure 2(a). All of these did not result from only the enzymatic cleavage in the liquid phase, but also from the use of Ficin without limitation in the quantity.

3.2. Comparison of Analytical Performances. Using the biotinylated preparations as the capture antibody, immuno-analytical systems were constructed to obtain the respective dose-response curve for evaluating the analytical performance of the antibody, in particular, the detection capability

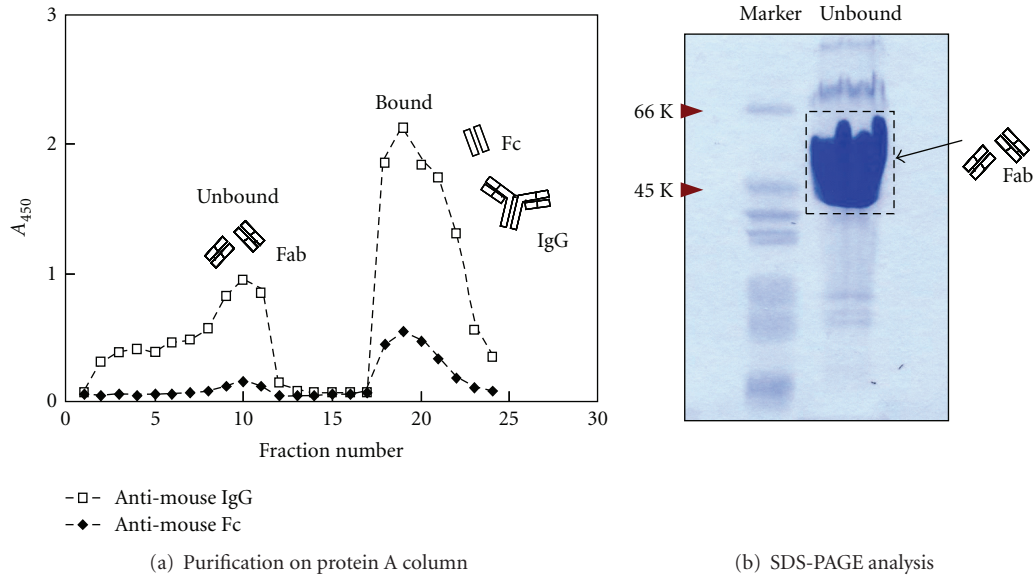


FIGURE 1: Affinity chromatography for the isolation of Fab from the antibody fragment mixture digested by Ficin protease and its identification. After loading the cleaved products on a protein A column, the unbound fractions were collected and the bound were eluted by shifting the medium pH. Each fraction was analyzed for total antibody and for that with Fc, which showed that the unbound included Fab and the bound consisted of fragments containing Fc (a). Furthermore, SDS-PAGE under non-reducing conditions confirmed that Fab was indeed the main component in the unbound portion. (b).

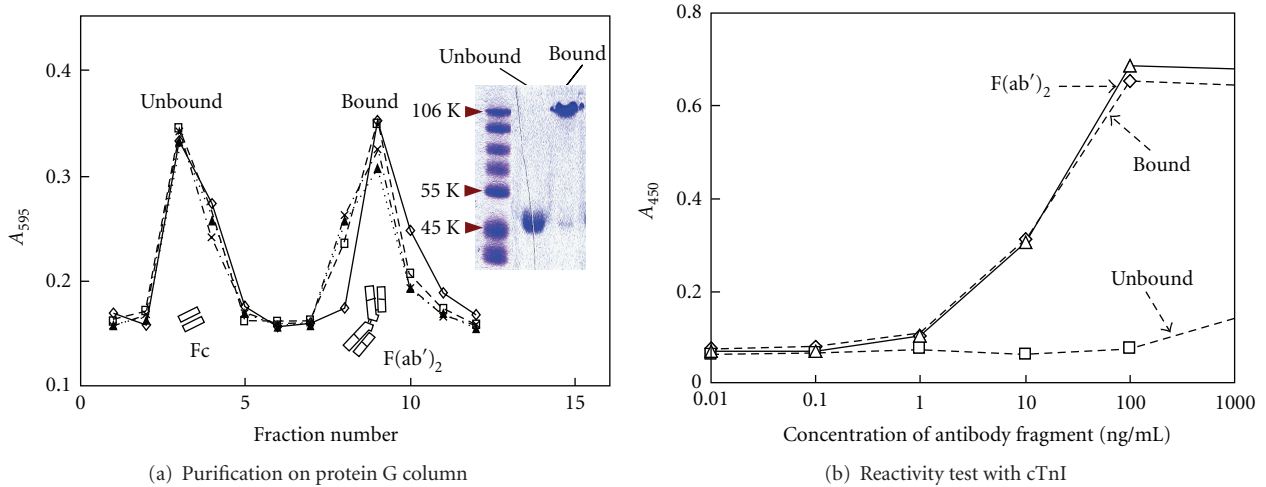


FIGURE 2: Affinity purification of the digested fragments of IgG type antibody on a protein G column and their characterization. Elution fractions from the column were analyzed for total protein by means of a Bradford assay (a). The fractions in each peak were pooled, which were then identified by molecular size via SDS-PAGE under non-reducing conditions (inset, (a)). The reactivities of each pooled fraction were subsequently confirmed by solid-phase immunoassays with cTnI as the antigen immobilized on the solid matrix (b)

(also expressed as sensitivity). The antibodies used in this study were highly specific in measuring analyte, that is, cTnI as a specific AMI marker, present in serum as a medium for clinical tests [16, 17]. Each antibody were immobilized on the inner surfaces of microwells in low and high densities to establish the binding models, and sandwich-type enzyme immunoassays were carried out to plot the signal against the cTnI concentration (Figure 3).

From such prepared dose-response curves, the detection limits for each antibody preparation were determined as

described previously [3]. With the antibodies immobilized at a low density (Figure 3(a)), the conventional, randomly biotinylated whole antibody showed the poorest sensitivity (detection limit = 1.826 ng/mL). When the site-directly biotinylated whole antibody was employed, the detection capability increased 6.1 times (detection limit = 0.297 ng/mL). Adoption of the antibody fragments, $F(ab')_2$ and Fab, further enhanced the sensitivity (0.205 ng/mL for the both). When the capture antibodies were immobilized at a high density (Figure 3(b)), the assay sensitivity with

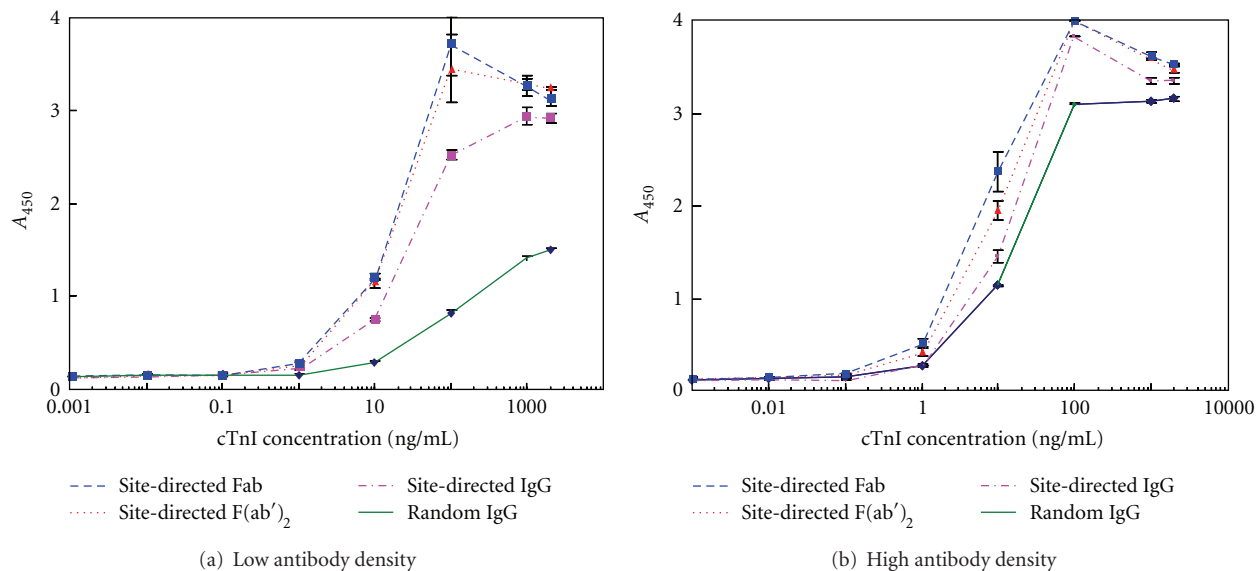


FIGURE 3: Dose-response curves for binding between each type of biotinylated antibody and cTnI at low (0.5 pmol/mL addition) and high (2.5 pmol/mL) surface densities of the respective antibody. The antibodies were immobilized via biotin-streptavidin linkage and used as the capture for carrying out sandwich enzyme immunoassays for cTnI. The signals produced from the assays were plotted against the analyte concentration. The mean of triplicate determinations for each measurement and the corresponding standard deviations were indicated.

the random preparation remarkably increased by 6.6 times (detection limit = 0.275 ng/mL) compared to immobilization at a low density. The site-directed products also showed somewhat improvements (detection limits = 0.246 ng/mL for IgG, 0.178 ng/mL for $F(ab')_2$, and 0.162 ng/mL for Fab), but the increments were relatively small. This may result from a change of the performance-controlling factor when the antibody density increased, which will be cleared in the rest of this paper.

Although the site-directly biotinylated fragments gave the best performances regardless of the surface density, a high density of the random IgG immobilization significantly enhanced the sensitivity. The fragmented products may have been immobilized in a controlled orientation as well as having flexible steric freedom and, consequently, had better accessibility to the antigen. This will be discussed in more detail below. As the surface density increases in the random preparation, the antibodies may change their molecular conformation into a more accessible orientation via self-assembly [2]. However, the use of a large quantity of antibodies as the capture may cause unwanted problems, for example, an increase in non-specific binding of the enzyme-labeled detection antibody to the immobilized layer [18] or negatively influencing the system [2, 3]. The site-directly prepared fragments immobilized at a 10 times lower density than the random IgG gave approximately an identical sensitivity. In this context, fragments with such performance would be preferable in most immunoassays that require a high signal-to-noise ratio as a precondition for enhanced detection capability.

3.3. Analyses of the Capture Antibody Performance. The complex formation of antigen with the immobilized antibody is affected mainly by three factors: method of immobilization,

surface density of the antibody, and size of antigen [2]. In general, these alter not only the density of reactive binding sites on the surfaces, but also the binding affinity of the antigen to the immobilized antibody. The number of reactive binding sites represents the status of the molecular orientation of the layer, and the affinity represents the steric freedom of the antibody accessible to the antigen. Therefore, these two parameters were used to analyze the improved performances of the site-directly biotinylated and suitably fragmented antibodies.

3.3.1. Surface Density of Reactive Binding Sites. To determine the reactive binding sites of the immobilized antibody, Scatchard analysis [13] can be carried out with a sandwich-type enzyme immunoassay using a microtiter plate for relatively easy measurements. The use of the detection antibody labeled with an enzyme (e.g., HRP), however, may sterically hinder binding between the antigen (e.g., cTnI) and the capture antibody. We performed these experiments and analysis based on the following four assumptions: (1) The binding sites on the surfaces are uniform for reaction with antigen; (2) Each antigen-antibody binding event is independent of one another; (3) Dissociation of the complex during incubation and washing is negligible; and (4) The detection antibody labeled with the enzyme covers all of the binding complexes such that one enzyme molecule represents a paired complex.

While the first three assumptions are usually applicable to any solid phase immunoassay, the fourth may be dependent on the quality of labeled detection antibody. The detection antibody used in this study was chemically coupled to HRP, which was analyzed by SDS-PAGE. From this analysis we found that the major portion (over 80%) of protein was indeed positioned at the 200 kDa band (data not shown).

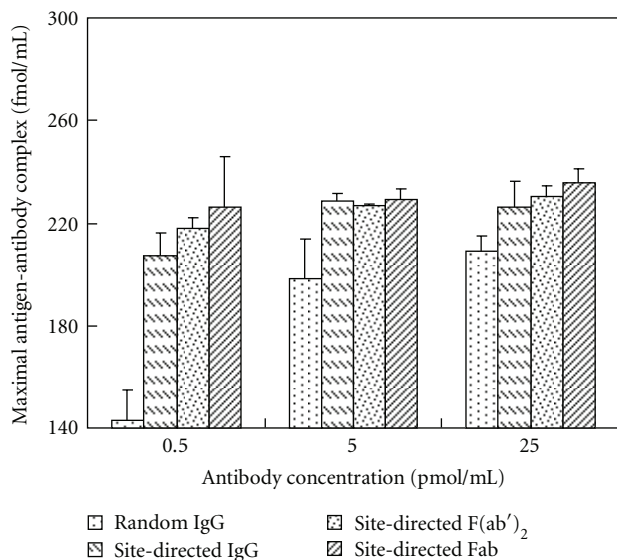


FIGURE 4: Comparison of the reactive antibody densities immobilized on the solid surfaces via biotin-streptavidin linkage. At a constant concentration of antibody, the reactive antibody density was determined by means of Scatchard analysis. The same procedure was repeated with different antibody preparations of IgG, $F(ab')_2$, and Fab. Standard deviations of triplicate repetition for each measurement were shown.

Since the molecular weights of the antibody and HRP are 155 and 44 kDa, respectively, the conjugation ratio on average should be very close to one. In addition, the three different types of antibodies, that is, whole IgG, $F(ab')_2$, and Fab, employed in this study had approximately same association/dissociation rate constants when measured using a surface plasmon resonance sensor system under the identical conditions. This indicated that the fragmentation of the antibody using protease did not alter the binding kinetic properties.

Under these assumptions, the surface density of the binding sites participating in the antigen-antibody reaction was determined by means of the Scatchard analysis for four different preparations of antibody: randomly or site-directly biotinylated whole IgG, site-directly prepared $F(ab')_2$, and site-directly synthesized Fab. The quantity of complex formation was estimated on the basis of the HRP activity coupled to the detection antibody, and the bound over the unbound antigen was plotted against the bound antigen concentration. Identical experiments were repeated at different antigen concentrations. For a Scatchard plot, a regression line was prepared to minimize errors between the values measured and estimated. The surface density of the binding sites and affinity was determined from the x-intercept and slope, respectively, of the Scatchard plot [13]. It is worth noting that the Scatchard plot was linear, indicating that the four assumptions proposed above were valid.

The density of binding sites available for the complex formation was determined at a constant concentration of each antibody, which was then replicated at different concentrations (Figure 4). At a low antibody concentration

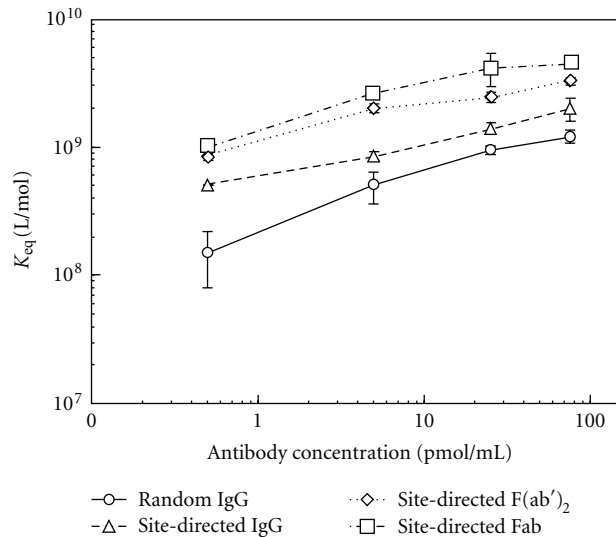


FIGURE 5: Comparison of binding affinities for the four different antibody preparations, as described in Figure 3, when plotted against the concentration added. The affinity was measured simultaneously with the surface density by means of Scatchard analysis. Each measurement were repeated three times and the resulting deviations were marked.

(e.g., 0.5 pmol/mL), the randomly biotinylated IgG showed a significantly low reactivity comparing to the site-directly prepared antibody products, although the total number of immobilized antibody was higher (data not shown). However, this gap decreased as the antibody concentration increased, which indicates that there was a gradual formation of a self-assembled molecular layer on the surfaces [2]. For the site-directed preparations, their relative densities were in an order of $Fab > F(ab')_2 > IgG$ at a low concentration, and became approximately the same but still greater than IgG as the concentration increased. These results indicate that the fragmented preparations essentially oriented the binding sites to face the bulk solution, but it may be very difficult for IgG to bind to the solid surfaces without impairing the binding sites for the reaction. There, however, could be subtle discrepancies between the fragmented species with regard to accessibility to the antigen present in solution, which will be further analyzed in terms of the binding affinity below.

3.3.2. Binding Affinity. As described above, the binding affinity of the immobilized antibody can also be determined concurrently with the reactive density of antibody binding sites from the Scatchard analysis. The binding affinities of four different types of the biotinylated antibody species were plotted against the antibody concentration added (Figure 5). Compared with the randomly immobilized IgG, the site-directly prepared antibodies (IgG, $F(ab')_2$, and Fab) showed high affinities over the entire antibody concentration range tested. Among the site-directed preparations, the relative affinities were consistent ($Fab > F(ab')_2 > IgG$) regardless

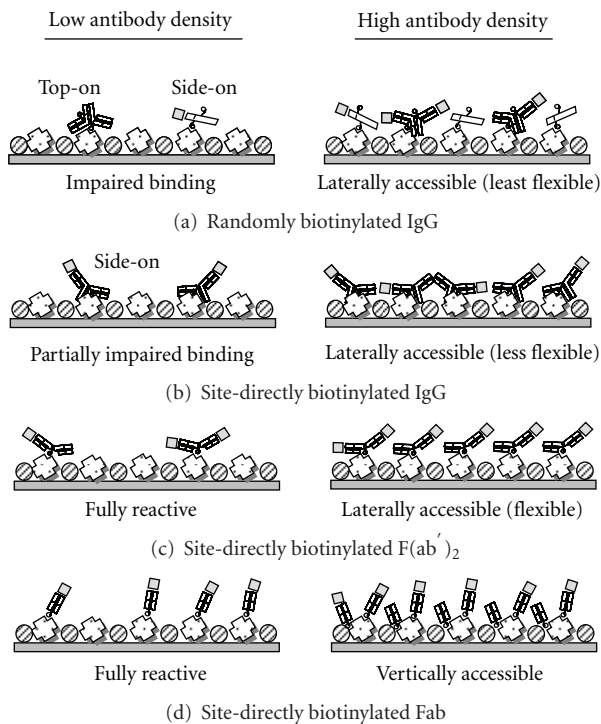


FIGURE 6: Equilibrium binding models of immobilized antibodies at solid surfaces. The models were devised on the basis of the variation patterns of the reactive antibody density (refer to Figure 4) and binding affinity (refer to Figure 5) against the antibody concentration for four different biotinylated preparations (see text for details). The major factor controlling the antigen-antibody complex formation seemed to be molecular orientation at a low antibody density (left) and accessibility at a high density (right).

of the concentration, suggesting formation of unique conformations to each antibody species upon insolubilization onto the matrix.

3.4. Determination of Performance-Controlling Factors. Conformations of each antibody preparation at the solid surfaces could be distinctive and determined mainly according to differences in the molecular shape (shown as IgG, $F(ab')_2$, and Fab) and biotinylation method (Figure 6). Among the antibodies tested, both of the native IgG type biotinylated using either the random Figure (6(a)) or site-directed method Figure (6(b)) showed the lowest binding affinities (refer to Figure 5), which may be linked to the presence of Fc. Although the Fc region has definite biological roles in vivo [19], this can hinder the attainment of a molecular orientation suitable for antigen-antibody binding when immobilized under an artificial environment. The IgG preparation did indeed seem to be present on the solid surface layer in a configuration, that is, “side-on”, with multiple contacts, which brought the binding sites of the molecules into the proximity of the surface. Thus, the surface can partially restrain the steric freedom of immobilized molecules required for antigen-antibody binding, resulting in a reduction of the binding affinity (known as surface

effect) [2]. Between the biotinylation methods, the random protocol resulted in the worse configuration (e.g., “top-on”) and was the major factor that reduced the number of reactive binding sites, particularly, when the surface density was low (refer to Figure 4).

The other two site-directly synthesized fragments without Fc, $F(ab')_2$ and Fab, which relieved the surface effect when used as the capture antibody (refer to Figure 5), are also different in their shape with respect to the number of binding units. The $F(ab')_2$ were immobilized via the hinge and sat on the surfaces in an orientation that turned the binding sites toward the bulk solution (Figure 6(c)). Since the molecules consisted of two binding sites located on the opposite ends of the left and right arms, the antigens in solution had to diffuse in a lateral direction to approach the sites to form the binding complex. In contrast, since the Fab fragment consisted of a single binding unit, it could be immobilized in an erect position where the binding site faced the bulk solution even though the Fab fragment was biotinylated on the same region (Figure 6(d)). In this case, the antigens can access the binding sites in the vertical direction, which may be more free of diffusion limitations caused by the surface layers present on the solid matrix.

The analyses done in this study show that factors controlling the performance of the capture antibody seem to be different according to the antibody density present on the solid surfaces. When the antibody density was low, orientational arrangement of the molecules mainly limited the number of binding sites for antigen in solution. Particularly, in case of randomly biotinylated IgG, the antibody may be immobilized mostly in “top-on” and “side-on” configurations, resulting in an inaccessible binding site. The site-directly prepared IgG may also have a “side-on” conformation due to the presence of the Fc region, which could decrease the accessibility of the antigen in solution. When the density increased, a molecular layer organization called self-assembly occurred and under such crowding conditions accessibility of the antigen to the binding sites could alter the binding affinity. In the case of antibody molecules with two associated binding units, the antigens can only laterally access the immobilized sites by diffusion. This process could be regulated by the molecular layer formed on the surfaces, which was more pronounced provided the steric freedom of the antibody was restricted by, for instances, the presence of Fc and immobilization via multiple biotin-streptavidin linkages.

4. Conclusions

Various antibody fragments, $F(ab')_2$ and Fab, biotinylated at the hinge region were produced and when used as the capture antibody in a sandwich immunoassay for cTnI displayed an enhanced analytical sensitivity that was about 6 times higher than the randomly biotinylated whole IgG under a low antibody density condition. This performance was comparable to that of the randomly prepared IgG immobilized at a 10 times higher density on the solid surfaces. Such a high detection capability with the scanty fragments could result mainly from a controlled conformation of the capture

antibody and also from the flexibility due to the absence of Fc, which offer conditions suitable for the binding complex formation. These features would be beneficial in immuno-analytical systems for the reduction of non-specific binding caused by protein-to-protein interactions or even by cross-reaction between antibody species employed as reagents.

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