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Site-directed Immobilization of Antibody onto Solid Surfaces for the Construction of Immunochip

Il-Hoon Cho[1], Eui-Hwan Paek[2], Haewon Lee[3], Jeong-Woo Choi[4], and Se-Hwan Paek[1,5]*

[1] Program for Bio-Microsystem Technology, Korea University, Seoul 136-701, Korea
[2] BioDigit Laboratories Corp., Technical Incubation Center, Biotechnology Building, Korea University, Seoul 136-701, Korea
[3] Department of Chemistry, Hanyang University, Seoul 133-791, Korea
[4] Chemical Engineering Department, Sogang University, Seoul 121-742, Korea
[5] Department of Biotechnology, Korea University, Jochiwon 339-700, Korea

Abstract The performance of an immuno-analytical system can be assessed in terms of its analytical sensitivity, *i.e.,* the detection limit of an analyte, which is determined by the amount of analyte molecules bound to the capture antibody that has been immobilized onto a solid surface. To increase the number of the binding complexes, we have investigated a site-directed immobilization of an antibody that has the ability to resolve a current problem associated with a random arrangement of the insolubilized immunoglobulin. The binding molecules were chemically reduced to produce thiol groups that were limited at the hinge region, and then, the reduced products were coupled to biotin. This biotinylated antibody was bound to a streptavidin-coated surface via the streptavidin-biotin reaction. This method can control the orientation of the antibody molecules present on a solid surface and also can significantly reduce the possibility of steric hindrance in the antigen-antibody reactions. In a two-site immunoassay, the introduction of the site-directly immobilized antibody as the capture enhanced the sensitivity of analyte detection approximately 10 times compared to that of the antibody randomly coupled to biotin. Such a novel approach would offer a protocol of antibody immobilization in order for the possibility of constructing a high performance immunochip.

Keywords: reduced immunoglobulin, antibody fragmentation, streptavidin-biotin linkage, two-site immunoassay, detection limit

INTRODUCTION

A protein chip is an analytical device that measures a number of different protein analytes at the same time [1-3], which may be similar to DNA chip in terms of its multi-analytical function. A simultaneous analysis of many analytes facilitates the acceleration of the information processing speed on particular targets. For instance, screening of donated blood samples against infectious diseases and comparing protein profiles in different biological specimen are the regimes utilized by the protein chip. Variable applications are feasible to a variety of fields, such as medical diagnostics, proteomic studies, monitoring of food and environmental contaminants, and veterinary examinations.

The protein chip selectively measures protein analytes by employing binding substances, and typically, these binding substances are antibodies that specifically recognize the respective analyte. Antibodies are complex organic molecules having globular folding of polypeptide chains and, thus, are sensitive and fragile under various conditions [1,2]. Furthermore, the antigen binding sites on the molecule are very small entities, each made of 3 to 5 amino acids, and far localized at Fv fragments of the immunoglobulin structure. When antibodies are immobilized on solid surfaces to construct an immunochip, their ability to bind to antigens may be significantly affected depending on the orientation of the immunoglobulin that is bound and the formation of molecular clusters on the surfaces [4]. For these reasons, it is important to develop appropriate immobilization methods for the immunoglobulin, if possible, without inactivation [3].

In general, most solid-phase immunoassays utilize antibodies that are immobilized onto solid substrates in undefined states [2,4]. This is caused mainly by a random, physical adsorption of the protein molecules onto the surface and also by mutual interactions among them by weak forces. These may result in the obstruction of suitable antigen-antibody reactions due to a steric hindrance as well as the creation of inactive binding sites directly facing the surface [2-4]. Such effects implicate that only a small fraction of the antibody molecules expose their

***Corresponding author**
Tel: +82-2-3290-3438 Fax: +82-2-927-2797
e-mail: shpaek@korea.ac.kr

binding sites to the outside surface to which the analyte present in the bulk solution can react. This problem occurs in most conventional immunoassays and causes decreases in analytical sensitivities.

It has been reported that antibody molecules can be controlled to be suitably immobilized onto a solid surface such that the antigen binding sites are oriented away from the surface [1]. Typically, chemical activation of the carbohydrate chain on the hinge region of the immunoglobulin can be performed using periodate oxidation in order for the binding protein to be attached to an activated surface. This type of immobilization may leave the Fab fragments free to interact with the antigen molecules [2]. Additional methods that utilize fragmented immunoglobulin are also available for controlled immobilization [5-7]. Nevertheless, no antibody immobilization method, to our knowledge, has yet presented solid experimental data showing a significant enhancement in the analytical sensitivity.

In this study, we mainly focused on the methods of controlling the orientation of the immunoglobulin when they are immobilized on solid surfaces. To help accomplish this purpose, reduction of the antibody molecules has been selected. Immunoglobulins can be reduced by a mild reducing agent such as mercaptoethylamine (MEA, also alternatively named as cysteamine). The resulting cysteine thiol groups, which are located at the hinge region of the antibody, can be used for the chemical attachment of the antibody fragments to an activated solid surface [8-10]. This can allow the antigen binding sites to face away from the surface, and consequently, this process increases the fraction of active antibody molecules. We compared the detection sensitivity of an analytical system that employing the approach under discussion with the detection sensitivity of a conventional method.

MATERIALS AND METHODS

Materials

A monoclonal antibody against human serum albumin (HSA) was obtained from BioDigit Laboratories (Seoul, Korea) and was purified on a protein G affinity column (Bio-Rad, Hercules, CA, USA). Goat anti-HSA polyclonal antibody was purchased from Cliniqa (Fallbrook, CA, USA) and was purified by immuno-affinity chromatography on a gel column with immobilized HSA. Rabbit anti-goat polyclonal antibody that was conjugated with horseradish peroxidase (HRP), Sephadex G-15 gel, CNBr-activated agarose gel, tetranitromethane, and mercaptoethylamine (MEA) were purchased from Sigma (St. Louis, MO, USA). N-hydroxysuccinimidyl (NHS)-biotin (Cat. #21343) and 1-biotinamido-4-(4'-[maleimidoethylcyclohexane]-carboxamido)butane (biotin-BMCC) (Cat. #21900) were purchased from Pierce (Rockford, IL, USA). Other reagents used in this study were of analytical grade.

Site-directed Biotinylation of Immunoglobulin

The monoclonal antibody to HSA was first dialyzed against a 100 mM phosphate buffer, pH 6.0, and was treated with variable concentrations of MEA (1 to 100 mM after dilution) as a reducing agent. After allowing the reaction to run for 90 min at room temperature, the excess MEA was removed by size-exclusion chromatography on a Sephadex G-15 gel column. The degree of the reduced state of immunoglobulin was verified by PAGE using a 12% gel. The samples were treated with a 60 mM Tris-HCl buffer of pH 6.8, which consisted of 25% glycerol, 2% sodium dodecyl sulphate, and 0.1% bromophenol blue. After loading the samples, the separation was run under 100 V and 40 mA for 90 min. The gel was stained with a 0.1% Coomassie Blue R-250 solution containing 45% methanol and 10% acetic acid for 1 h, and then, the gel was destained.

Using an optimal concentration of MEA (50 mM), the reduced antibody was immediately reacted with a 20 molar excess of biotin-BMCC that had been dissolved in dimethyl sulfoxide for 2 h at room temperature. Unreacted biotin molecules were removed by dialysis and subsequently by size-exclusion chromatography.

Purification of Biotinylated Immunoglobulin

To prepare a nitro-avidin gel column, avidin was dissolved in a 50 mM carbonate buffer of pH 8.0, and tetranitromethane (0.4 μL/mg avidin) was then added into the solution. The reaction mixture was incubated within a dark box for 1 h at room temperature. This nitrated avidin product was added to a CNBr-activated agarose gel that was prepared according to the manufacturer's protocol. After reacting the mixture at 4 overnight, the avidin-immobilized gel was extensively washed with the carbonate buffer. Then, the gel was transferred into a 100 mM Tris buffer of pH 7.6 to inactivate the residual reactive groups, and it was filled within a glass column (gel volume, 7 mL). After washing the gel by alternating acidic and alkaline conditions, 0.6 mM biotin dissolved in 50 mM sodium acetate buffer (pH 4) was added to block unmodified biotin-binding sites of the immobilized avidin. Biotin molecules that were bound to the nitro-avidin were released using a 50 mM carbonate buffer of pH 10. Finally, the gel column was washed with a sufficient amount of 50 mM sodium acetate buffer (pH 5.0).

In order to purify the biotinylated antibody prepared above, the antibody was loaded into the nitro-avidin gel column, and the gel was subsequently washed with the 50 mM sodium acetate buffer (pH 5.0). The eluate from the column was delivered at a rate of 2 mL/h to a fraction collector (Model 2110, Bio-Rad, Hercules, CA, USA) and was collected in a fraction volume of 1 mL. After sufficient washing, the bound antibody was eluted by switching the buffer to a 50 mM carbonate buffer of pH 10. The fractions collected were subjected to analyses as described below.

Analyses of Elution Fractions

The elution fractions from the affinity column were analyzed for protein, total antibody, and the biotinylated

antibody. Protein analyses were carried out using the Bradford assay [11]. Total antibody assays were accomplished using a goat anti-mouse antibody that was immobilized within the inner surfaces of the microwells. The goat antibody (1 μg/mL), which was diluted in a 10 mM phosphate buffer containing 140 mM NaCl (pH 7.4) (phosphate buffered saline, PBS), was added into the wells (100 μL each) and was incubated for 1 hr at 37°C. After washing, the residual surfaces were treated with 100 mM Tris-HCl of pH 7.6 containing 0.5% casein (Tris-Casein). The samples, which were diluted to 1:100 with Tris-Casein containing 0.1% tween-20 (Tris-Casein-TW), were added into each antibody-immobilized well and reacted under the same conditions. A goat anti-mouse polyclonal antibody coupled to horseradish peroxidase (HRP), which was diluted in Tris-Casein-TW, was transferred after washing and was incubated under identical conditions. The wells were rinsed, a substrate solution for HRP [12] was added, and the color that developed was measured at the absorbance of 450 nm. Assays for the biotinylated antibody were performed employing the same analytical protocol employed for the total antibody except the use of microwell with immobilized streptavidin instead of the secondary antibody.

Dose responses in Two-site Immunoassays

To compare the performances of two different antibody preparations, *i.e.*, site-directly biotinylated antibody and randomly biotinylated antibody, immunoassays on streptavidin-coated surfaces were performed. The randomly biotinylated antibody as the control was synthesized by coupling 20 molar excess of NHS-biotin to the intact antibody. The excess biotin was then removed by dialysis and gel filtration. For immobilization of the antibodies, streptavidin (10 μg/mL) dissolved in PBS was coated on the inner surfaces of the microwells that were already treated with 0.5% glutaraldehyde solution. The wells were incubated within a closed box, and 100% humidity was maintained for 1 h at 37°C. These same conditions were also used for incubations in the following steps that are mentioned. After washing, the residual surfaces were blocked with Tris-Casein, and each antibody preparation (2 μg/mL), which was dissolved in Tris-Casein-TW, were immobilized on separate streptavidin-coated surfaces of the microwells. Variable concentrations of HSA (0 to 1 μg/mL) were bound within different wells for each set of the capture antibody. Goat anti-HSA polyclonal antibody (1 μg/mL), which was used as the detection antibody diluted in Tris-Casein-TW, was allowed to react in each well, and rabbit anti-goat polyclonal antibody coupled to HRP was subsequently bound. After rinsing, a substrate solution for HRP [12] was added, and a color signal that developed was measured as described above.

RESULTS AND DISCUSSION

To attain an efficient immobilization of immunoglobulin, we have investigated a defined activation of the antibody molecule in combination with an immobilization method that employs streptavidin-biotin linkage. This would provide two advantages. First, the utilization of a streptavidin-coated solid support enables the blockage of the residual surfaces with an inert protein (*e.g.*, casein, bovine serum albumin) after immobilizing the binding molecules. Such blocking can prohibit a direct contact of the immunoglobulin to the solid surface, which causes a non-specific adsorption of the protein [2]. Secondly, the sites of biotinylation on the antibody molecule can be selective such that a site-directed immobilization of immunoglobulin can be accomplished using streptavidin-biotin binding. The sites for the attachment of biotin are possibly the sulfhydryl groups that were produced by chemical reduction at the hinge region of the antibody molecule.

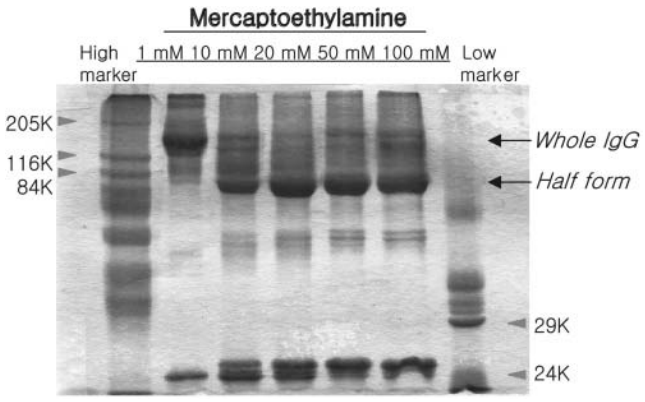

Fig. 1. SDS-PAGE analysis of the antibody molecules reduced using variable concentrations of MEA. The reductions were carried out at 37°C for 90 min in the presence of MEA that had a concentration between 1 and 100 mM.

Preparation of Immunoglobulin Biotinylated at a Defined Site

Strong reducing agents, such as dithiothreitol and mercaptoethanol, appeared to cleave disulfide bonds that were present between the heavy and light chains as well as the target between the heavy chains of the antibody molecule. This resulted in a production of sulfhydryl groups at unwanted sites, which may interfere with the formation of a reproducible, uniform antibody layer on a solid surface and also caused a denaturation of the protein. Thus, we introduced MEA for a mild reduction of the antibody molecule, which lead to the selective cleavage of the disulfide bonds at the site between the heavy chains.

Optimization of Reduction Condition

Fig. 1 shows the results of the SDS-PAGE analysis of the antibody molecules reduced by MEA. Although some protein bands were observed at a range of low molecular weight (near MW 24,000), which indicate the cleavage of light chains, the major protein was the half form (near

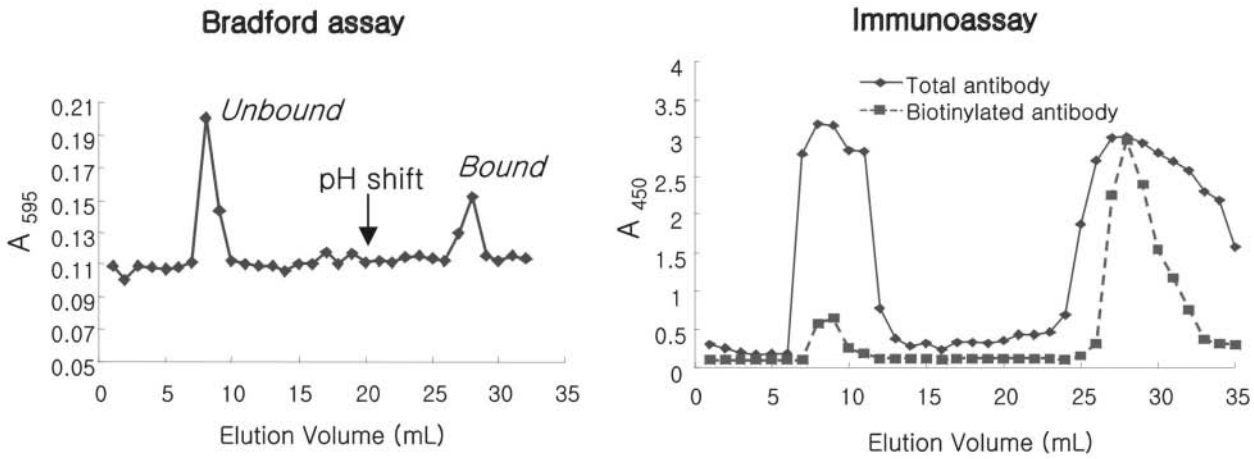

Fig. 2. Affinity purification of the biotinylated antibody on a nitro-avidin gel column. Bradford assay was performed for each eluted fraction (left), and immunoassays for total and biotinylated antibodies containing in each fraction (right) were performed.

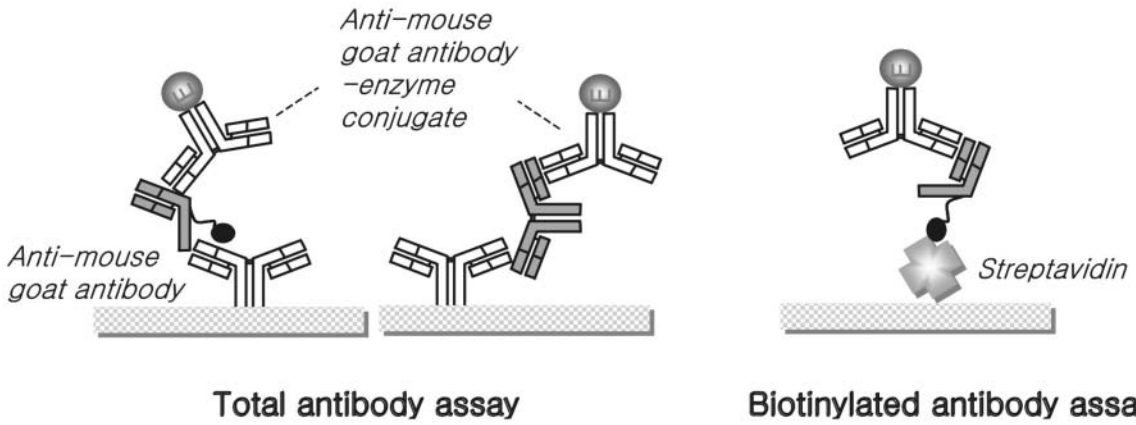

Fig. 3. Immunoassays for total and biotinylated antibodies to identify the constituents in the peaks shown in Fig. 2.

MW 84,000) of immunoglobulin if MEA was sufficiently used for the reaction (equal to or more than 10 mM). This demonstrated that MEA reduced the disulfide bridges present between the heavy chains rather than those existent between the light and heavy chains. Such activated thiol groups on the protein molecules can be preserved by adding ethylenediaminetetraacetic acid as an anti-oxidative agent until they are utilized for the coupling with biotin [13]. Under this condition, more than 90% of the thiol groups have been reported to be reactive over a 40 h incubation period.

Preparation of Biotinylated Antibody

The fragmented antibodies with active thiol groups on the hinge region were chemically linked to the biotin molecules that were activated to produce maleimide groups. The resulting biotinylated antibody allows for an oriented attachment of the antibody on a streptavidin-coated solid surface via streptavidin-biotin binding. To prepare a defined analytical component, the biotinylated antibody was purified by affinity chromatography on a nitro-avidin gel column (Fig. 2). After loading the reaction mixture onto the column, the unbound fraction was sufficiently washed with an excess amount of a neutral pH buffer and the bound fraction was subsequently eluted under a basic condition (*e.g.*, pH 10) that significantly reduced the binding affinity of nitrated avidin toward biotin [14]. The Bradford assays of the eluents for the total protein identified two peaks (Fig. 2, left) that possibly contained different proteins toward the state of conjugation: free antibody in the first peak and the biotinylated in the second. To support these estimates, immuno-analyses of each fraction for total antibody and the biotinylated antibody were performed (see Fig. 3 for each scheme). The results showed that the antibody was present in both peaks while the biotinylated antibody was detected only in the second peak (Fig. 2, right). The small first peak found in the biotinylated antibody assay could have resulted from the non-specific binding of the protein.

Because the avidin-biotin reaction occurred with an extremely high affinity constant [14], one of the major drawbacks in utilizing the reaction as a tool for purifica-

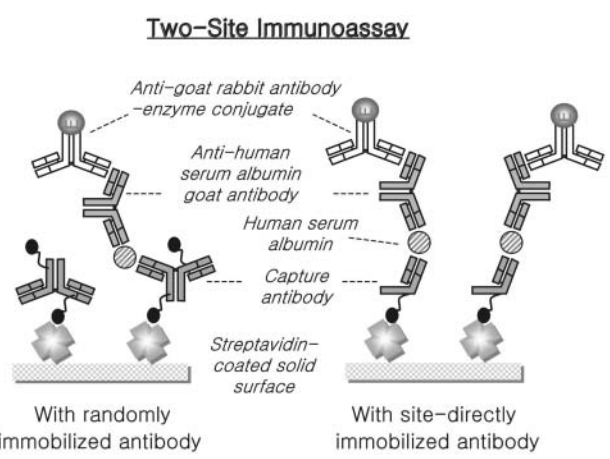

Fig. 4. Schematic presentation of conceivable configurations of sandwich immune complexes formed in the respective immunoassay system with different capture antibodies. One was the antibody biotinylated at random positions (left) while the other at the defined hinge region (right).

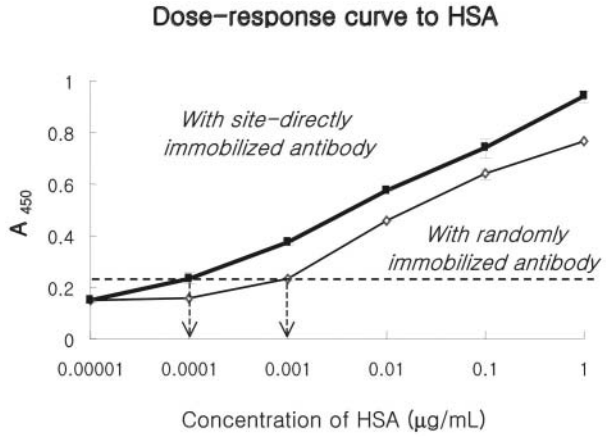

Fig. 5. Comparison of the performances of two different capture antibody preparations in a two-site immunoassay, *i.e.*, site-directly immobilized antibody and randomly immobilized antibody on streptavidin-coated surfaces. Variations of duplicate measurements are shown.

tion of the biotinylated antibody was a nearly irreversible nature of the complex formation [15]. In order to disrupt the complexes from being formed, a condition of denaturation may be required [16]. Such drastic conditions would invariably inactivate the biological activity of the biotinylated antibody, and thus, an alternative approach of purification was necessary in order to prepare the antibody suitable for subsequent uses. The binding sites on the avidin molecule contained tyrosine residue that could be modified at its *ortho* position by nitration to synthesize a new component, nitro-avidin. Unlike the native form, such a derivatized avidin exhibited characteristics of a reversible dissociation from the complex with biotin at an elevated pH condition [14]. Utilizing such an effect, we were able to highly purify the biotinylated antibody without the occurrence of denaturation by using the gel column containing immobilized nitro-avidin as shown above.

Assessment of Analytical Performance of the Biotinylated Antibody

Since the biotinylated antibody prepared will be eventually adopted as an immuno-analytical component, we tested its performance, particularly, in a two-site immunoassay. To this end, the biotinylated antibody was immobilized on a streptavidin-coated surface and preparations were made to obtain the dose-response curve of HSA as an analyte. The effect of biotinylation at the defined sites of the immunoglobulin could enable us to immobilize them in a controlled fashion on a streptavidin-coated layer, which was tested by comparing its performance with one that was conventionally prepared (see Fig. 4 for configurations of each analytical system). The conventional system used as a control in this study employed the intact antibody conjugated with an amine-reactive biotin, NHS-LC-LC-Biotin. Such a prepared antibody would have multiple biotin molecules randomly bound on an immunoglobulin, which may result in variable orientations of the immobilized antibody on the streptavidin-coated surface. This may reduce the active binding sites accessible to an antigen due to steric hindrance.

The performance of the site-directly biotinylated antibody was compared as the capture agent in the dose-response curve with that of the randomly biotinylated antibody (Fig. 5). The results revealed that at least an increase of sensitivity by 10 fold (*i.e.,* detection limit of analyte concentration corresponding to the signal value calculated by multiplying the standard deviation at the zero dose by three) was indeed obtained with the site-directly biotinylated antibody. Unlike the randomly arranged antibody on the solid surface, the capture antibody that was immobilized in a defined fashion provided a high yield of reactive antigen-binding sites that faced away from the surface as mentioned. Consequently, the introduction of the site-directed immobilization method can significantly reduce steric hindrance in the antigen-antibody binding compared to the steric hindrance with the conventional. In addition, the utilization of a streptavidin-coated surface for an antibody binding can offer a maximum packing density of immunoglobulin [2].

In conclusion, a method for site-directed immobilization of an antibody via streptavidin-biotin linkage on a solid surface, which results in a significant enhancement in the analytical sensitivity, was developed. We attribute such results to an increased yield of active binding sites of immunoglobulin arranged in an orderly manner on the surface. This would resolve one of the major problems in constructing a high performance immunochip, *i.e.*, random arrangement of immunoglobulin. We are further challenging, for a better performance of immunochip, the investigation of a method that prevents the antibody molecules from forming protein molecular clusters on the surface.

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REFERENCES

[1] Lu, B., M. J. Smyth, and R. O'kennedy (1996) Oriented immobilization of antibodies and its applications to immunoassays and immunosensors. *Analyst.* 121: 29R-32.

[2] Peluso, P., D. S. Wilson, D. Do, H. Tran, M. Vendatasubbaiah, D. Quincy, B. Heidecker, K. Poindexter, N. Tolani, M. Phelan, K. Witte, L. S. Jung, P. Wagner, and S. Nock (2003) Optimizing antibody immobilization strategies for the construction of protein microarrays. *Anal. Biochem.* 312: 113-124.

[3] Wilson, D. S. and S. Nock (2001) Functional protein microarrays. *Curr. Opin. Chem. Biol.* 6: 81-85.

[4] Schramm, W. and S. H. Paek (1992) Antibody-antigen complex formation with immobilized immunoglobulin. *Anal. Biochem.* 205: 47-56.

[5] DeSilva, B. S. and G. S. Wilson (2000) Synthesis of bifunctional Antibodies for iImmunoassays. *Methods* 22: 33-43.

[6] Wang, H., Y. Liu, Y. Yang, T. Deng, G. Shen, and R. Yu (2004) A protein A-based orientation-controlled immobilization strategy for antibodies using nanometer-sized gold particles and plasma-polymerized film. *Anal. Biochem.* 324: 219-226.

[7] Brogan, K. L., K. N. Wolfe, P. A. Jones, and M. H. Schoenfisch (2003) Direct oriented immobilization of F(ab) antibody fragments on gold. *Anal. Chim. Acta* 496: 73-80.

[8] Lu, B., J. Xie, C. Lu, C. Wu, and Y. Wei (1995) Oriented immobilization of Fab' fragments on silica surfaces. *Anal. Chem.* 67: 83-87.

[9] Prisyazhnoy, V. S., M. Fusek, and Y. B. Alakhov (1988) Synthesis of high capacity immunoaffinity sorbents with oriented immobilized immunoglobulins or their Fab' fragments for isolation of proteins. *J. Chromatogr.* 424: 243-253.

[10] Jimbo, Y. and M. Saito (1988) Orientation-controlled immobilization of protein molecules on thin organic films deposited by the plasma technique. *J. Mol. Electron.* 4: 111-118.

[11] Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the priciple of protein-dye binding. *Anal. Biochem.* 72: 248-254.

[12] Paek, S. H., L. G. Bachas, and W. Schramm (1993) Defined analyte-enzyme conjugates as signal generators in immunoassays. *Anal. Biochem.* 210: 145-154.

[13] Riddles, P. W., R. L. Blakeley, and B. Zerner (1983) Reassessment of Ellman's reagent. *Method Enzymol.* 91: 49-60.

[14] Morag, E., E. A. Bayer, and M. Wilchek (1996) Immobilized nitro-avidin and nitro-streptavidin as reusable affinity matrices for application in avidin-biotin technology. *Anal. Biochem.* 243: 257-263.

[15] Wilchek M. and E. A. Bayer (1989) *Protein Recognition of Immobilized Ligands*. pp. 83-90. A.R. Liss, NY, USA.

[16] Green, N. M. (1963) Avidin: The nature of the biotin-binding site. *Biochem. J.* 89: 609-620.

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