Nano-Scale Immobilization of Antibody for the Construction of Immunosensor

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Abstract

Performance of an immunosensor can usually be assessed in terms of its analytical sensitivity and specificity. Sensitivity, i.e., the detection limit of analyte, is particularly determined by the amount of analyte molecules bound to the capture antibody immobilized onto a solid surface. In order to increase the binding complexes, we have investigated an immobilization method of antibody allowing for a molecular arrangement of the protein on a selective surface of a nano-patterned solid substrate. This has not been accomplished only by a surface treatment with a chemical, but also by fragmentation of immunoglobulin. Such approach would offer a protocol of antibody immobilization for the construction of nano-immunosensor and eventually improve the sensitivity of detection.

Introduction

Most immuno-analytical systems require one of the antibodies to be immobilized onto a solid surface. This can be simply accomplished by physical adsorption of the capture antibody onto, for an instance, a plastic surface of microtiter plate. However, this method has a problem, i.e., confirmational change of immunoglobulin upon binding to the surface.[1),2)] Moreover, proteins tend to mutually interact through weak forces including ionic interaction, hydrogen bonding, and van der Waals forces, which may lead to a formation of clusters on the surface. These problems cause a decrease in the analytical performance of an immunosensor.

In a direction to overcome such technical limitations, we have investigated a site-directed immobilization of immunoglobulin via streptavidin (SA)-biotin linkage. This can be readily

carried out by chemically reducing the antibody molecule and the resulting thiol groups are used to attach an activated biotin. The molecule derivatized with biotin is then bound onto a SA-coated solid surface. This method would significantly reduce the chance of steric hindrance when antigen-antibody reaction occurs on a given solid matrix[3] and also control the orientation of the antibody molecule bound[4]. The immobilization yield can be enhanced provided an enzymatically digested immunoglobulin, such as Fab, Fab', and $F(ab')_2$, is employed.

The other direction of this research is prepare an immobilization method of protein onto a selective surface. We have investigated a surface modification by treating it with a chemical preventing the surface from physical adsorption of protein. Hence, using this setting, a protein can be immobilized purely by covalent binding. We are currently using a nano-patterned surface containing activated areas toward chemical reaction such that a cluster formation of antibody molecules is minimal.

Materials and Methods

Pepsin (P-6887) and mercaptoethylamine (M-9768) for modifying immunoglobulin were purchased from Sigma (St. Louis, MO, US). A monoclonal antibody (MAb) to human serum albumin (HSA) was raised using a standard protocol, produced in a ascitic fluid from mice, and then purified on a protein G column. Silicon wafer (P-type, 100), gold-patterned in a square shape, was cut by a diamond cutter and then used for the immobilization of antibody.

Fragmentation of immunoglobulin

MAb produced was used as capture antibody in an immuno-analytical system. The antibody was modified using a proteolytic enzyme, pepsin, or a mild chemical reducing agent, mercaptoethylamine (MEA). The antibody was first dialyzed against 100 mM acetate buffer, pH 4.5, at room temperature for adjusting the reaction condition. To treat the antibody with pepsin, pepsinolysis was performed by reacting 40 excess enzyme in mass over the antibody at 37℃ for 2 h. The degree of fragmentation was analyzed using SDS-PAGE and ELISA after the pepsinolysis. For

chemical reduction, the antibody was treated with MEA as a mild reducing agent that cleaved the disulfide bonds present at the hinge region of the molecule. The antibody was dialyzed against 100 mM phosphate buffer, pH 6.0, containing EDTA and then treated with 50 mM MEA in the same buffer for 90 min at 37℃. After reaction, an excess MEA was removed on a desalting column and subsequently treated in SDS solution.

Immobilization of protein on a selected surface

Gold-patterned silicon substrate was cleaned by 1 M HCl for 10 min and then dried on a stream of nitrogen gas. The substrate was immersed in a detergent solution for 1 h. Streptavidin was activated with N-succinimidyl-3-

(2-pyridyldithio) propionate (SPDP) and reduced by a reducing agent, dithiothreitol, for 30 min. This activated protein was incubated on the detergent-coated substrate, and the substrate was blocked with 0.5% casein dissolved in tris buffer for 1 h. Biotinylated antibody was added to bind via SA-biotin linkage and a secondary antibody-horseradish peroxidase (HRP) conjugate was subsequently incubated. A chromogenic substrate for HRP was finally added for signal generation.

Results and Discussion

To enhance immuno-analytical performances, we conducted research in two directions, i.e., fragmentation of immunoglobulin for site-directed immobilization and immobilization of the protein on a selected surface patterned in a nano-scale.

Fragmentation of immunoglobulin for site-directed immobilization

For modification of antibody molecule, MEA was selected for reducing the protein to dissociate into a half form. If such reduced antibody was used to conjugate with biotin and subsequently immobilize via SA-biotin linkage, an increase in the sensitivity of immunosensor was expected.

MAb reduced by MEA was analyzed on SDS-PAGE, which indicated that the

concentration of MEA between 20 and 50 mM was appropriate. Another reducing agent, dithiothreitol (DTT), gave similar results to those with MEA in cleaving the disulfide bonds present at the hinge region of the antibody molecule. However, DTT resulted in a side reaction dissociating the light and heavy chains. Therefore, MEA was more suitable agent in preserving the activity of fragmented immunoglobulin after reduction.

The reduced antibody was utilized to chemically couple to a biotin derivative and subsequently to immobilize on a solid surface using SA-biotin binding. We obtained a dose-response curve to analyte (i.e, human serum albumin in this study) and compared the results with those from a conventional system where the intact MAb was immobilized by physical adsorption. This showed that the sensitivity defined as detection limit of analyte concentration was significantly higher in the system with the fragmented antibody than in the conventional.

Immobilization of the protein on a selected surface

In the other research, SA was thiolated and then coated on a gold surface to achieve a covalent immobilization. In this process, we encountered a problem that the modified protein tended to bind an inert surface (e.g., silicon) as well as the gold. To overcome such a problem, we investigated a method of introduction of barrier (e.g., pre-coating of detergent) against physical adsorption on the surface prior to the protein coating.

Streptavidin, which is the first biomolecule in this immunoassay system, is likely to adsorb not only on gold but silicon surface. To immobilize streptavidin specifically on gold except silicon we treated 5% tween-20 for 1 hour after cleaning the surface. After this step, thiolated streptavidin conjugated with SPDP and reduced by 1 mM DTT previously was perfomed, for chemical bond between streptavidin and gold surface. Fig. 1. shows the experimental results revealing the difference between the absence of the barrier and the presence on a gold-patterned silicon surface. Therefore, we are currently expanding this technology for the immobilization of immunoglobulin on a nano-patterned solid surface.

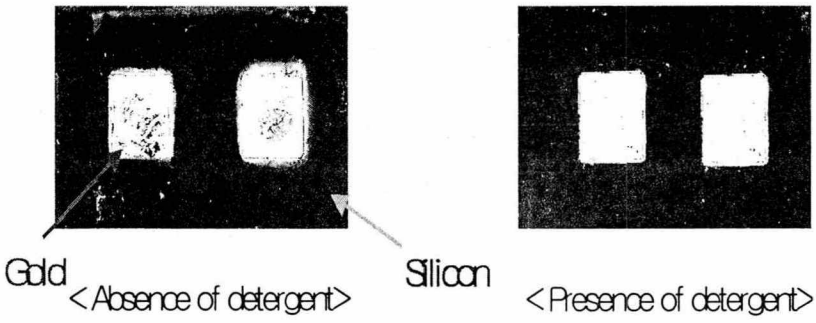

Fig. 1. Selective immobilization of SA on the surfaces without pre-coating of a detergent (left) and with the pre-coating (right) on a gold-patterned silicon surface.

References

1. J. E. Butler, L. Ni, R. Nessler, K. S. Joshi, M. Suter, B. Rosenberg, J. Chang, W. R. Brown, L. A. Cantarero (1992), The physical and functional behavior of capture antibodies adsorbed on polystyrene, *J. Immunol. Methods* **150**, 77-90.
2. J. E. Butler, L. Ni, W. R. Brown, K. S. Joshi, J. Chang, B. Rosenberg, E. W. Voss (1993), The immunochemistry of sandwich ELISAs.VI. Greater than 90% of monoclonal and 75% of polyclonal anti-.uorescyl capture antibodies (CAbs) are denatured by passive adsorption, *Mol. Immunol.* **30**, 1165-1175.
3. C. A. Rowe, S. B. Scruggs, M. J. Feldstein, J. P. Golden, F. S. Ligler (1999), An array immunosensor for simultaneous detection of clinical analytes, *Anal. Chem.* **71**, 433-439.
4. Paul Peluso, David S. Wilson, Duc Do, Huu Tran, Maanasa Venkatasubbaiah, David Quincy (2003), Optimizing antibody immobilization strategies for the construction of protein microarrays, *Anal. Biochem.* **312**, 113-124.