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A comparative study on antibody immobilization strategies onto solid surface

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Abstract−Antibody immobilization onto solid surface has been studied extensively for a number of applications including immunoassays, biosensors, and affinity chromatography. For most applications, a critical consideration regarding immobilization of antibody is orientation of its antigen-binding site with respect to the surface. We compared two oriented antibody immobilization strategies which utilize thiolated-protein A/G and thiolated-secondary antibody as linker molecules with the case of direct surface immobilization of thiol-conjugated target antibody. Antibody immobilization degree and surface topography were evaluated by surface plasmon resonance and atomic force microscope, respectively. Protein A/G-mediated immobilization strategy showed the best result and secondary antibody-mediated immobilization was the worst for the total immobilization levels of target antibodies. However, when considering real-to-ideal ratio for antigen binding, total target antigen binding levels (oriented target antibody immobilization levels) had the following order: secondary antibody-mediated immobilization>protein A/G-mediated immobilization>direct thiol-conjugated immobilization. Thus, we confirmed that protein A/G- and secondary antibody-mediated strategies, which consider orientation of target antibody immobilization, showed significantly high antigen binding efficiencies compared to direct random immobilization method. Collectively, the oriented antibody immobilization methods using linker materials could be useful in diverse antibody-antigen interaction-involved application fields.

Key words: Antibody Immobilization, Orientation, Thiolation, Protein A/G, Secondary Antibody, Surface Plasmon Resonance, Atomic Force Microscopy

INTRODUCTION

Antibody is the immune system-related protein called immunoglobulin (IgG). An antibody consists of four polypeptides; two heavy and two light polypeptide chains which are linked by disulfide bond to form a 'Y' shaped molecule. The amino acid sequence in the tips of the 'Y' greatly varies among different antibodies. This variable region gives the antibody its specificity to antigen. Treating antibody with a protease can cleave it into two parts, making an antigen binding fragment (F_{ab}) that includes the variable ends of an antibody and a well conserved crystallizable fragment (F_c) [1].

Effective antibody immobilization on solid surface is an important subject in various fields including immunoassays and immunosensors. Immunoassays have extensive applications in all areas of clinical and pharmaceutical chemistry as well as in environmental analysis [2]. Immunosensors generally have excellent advantages of accuracy, reproducibility, sensitivity, and cost [3,4]. Antibody immobilization also has applications in immune-affinity purification of proteins, enzyme reactors, and flow injection assays. The oriented immobilization of antibodies is considered to be a determinant of their effectiveness. An antibody is considered to be properly oriented and perfectly active when the F_c region, which has no antigen binding affinity, is immobilized on a surface rather than antigen-binding sites [5]. Many studies reported that antibody binding on a surface with its active F_{ab} sites prevents efficient antigen binding and biological activity. Thus, specific orientation is necessary to ensure binding site activity of immobilized target antibody. It was claimed that randomly immobilized antibodies have lower antigen-binding capacities than oriented immobilized antibodies [5-9]. In addition, specific orientation of target antibody was found to improve the sensitivity of a biosensor [7].

Several techniques for the immobilization of antibodies have been reported, and they can be divided into two groups: physical and chemical immobilization. In early days, target antibodies were directly immobilized onto solid surfaces by these methods. However, these direct immobilization methods highly reduced the antigen-binding ability due to random orientation and/or denaturation [5-9]. Therefore, antibody-immobilizing linker-based antibody immobilization methods have been employed to overcome the limitations of direct antibody immobilization methods. Antibody-binding proteins such as protein A/G and secondary antibody that can recognize F_c region of target antibody [10] have been known as representative antibody-immobilizing linkers [11,12].

In the present work, we compared two oriented antibody immobilization strategies using protein A/G and secondary antibody with direct thiol-conjugating strategy through analyses of surface plasmon resonance (SPR) and atomic force microscopy (AFM) to clarify more efficient and promising antibody immobilization method for

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practical applications.

MATERIALS AND METHODS

1. Thiol Group Introduction for Immobilization of Biomolecules on Gold Surface

To immobilize the biomolecules (monoclonal anti-green fluorescent protein (GFP) IgG (molecular weight ~150 kDa; Roche Diagnostics GmbH) as model target antibody for direct random immobilization and protein A/G (molecular weight ~50 kDa; Pierce) and monoclonal anti-mouse IgG (Sigma) as linkers for oriented immobilization) on gold surface for SPR analyses, thiolation was carried out in 2 mM phosphate buffered saline (PBS; pH 8.0) using 20 μM 2-iminothiolane (Pierce). For efficient thiolation, the samples were mixed gently and incubated for 1 h at room temperature. Next, the thiolated-protein A/G and -antibodies were purified by filtrations using 10 kDa and 50 kDa molecular cutoff polyethersulfone membranes (Vivaspin; Vivascience), respectively. Finally, the purified thiolated-biomolecule samples were stored on ice.

2. SPR Analysis

All SPR experiments were conducted with a Biacore 2000 instrument (GE Healthcare). Electrolyte (0.135 M NaCl, 1% (v/v) Tween-80, 2 mM PBS, 1% (v/v) Tween-20; pH 8.0) was prepared by filtering and degassing. We added Tween-20 in the electrolyte to block non-specific binding [13]. Bare gold surface (SIA Kit Au) was used to study interactions between surface materials and biomolecules. SPR was first stabilized by electrolyte during 5 min. Each sample was flowed into flow cells (channels 2-4), while channel 1 (control channel) was used as a baseline. Each flow rate was 5 L/min. 1 M samples including anti-mouse IgG, anti-GFP IgG, and GFP (molecular weight ~27 kDa; Clontech) were flowed into the SPR cell for 10 min, followed by flushing with electolyte for 5 min to remove reversibly bound proteins.

3. AFM Analysis

AFM (Digital Instruments) was operated in tapping mode. A commercial Silicon AFB probe (Tap300, BudgetSensors) with 40 N/m spring constant was used to perform the experiments. All sample preparation steps were the same as those in SPR experiments except the sample reaction time (longer than SPR measurement). All the experiments were performed in air at room temperature with relative humidity less than 40%. AFM image and corresponding cross section were calculated by means of the Scanning Probe Image Processor software (Image Metrology).

RESULTS AND DISCUSSION

1. Target Antibody Immobilization Strategies on Gold Surface

2-Iminothiolane, which is a cyclic thioimidate compound for thiolation [14], reacts with primary amines (-NH_2) of protein to introduce thiol (-SH) groups, while maintaining charge properties similar to the original amino group. Through thiolation, target biomolecules including antibody can be directly immobilized on gold surfaces effectively. However, due to its random-manner immobilization (Fig. 1(a)), direct target antibody immobilization method can have a limitation on lower antigen-binding capacity [5-9].

Protein A is the most successful surface protein able to bind with animal IgGs, but is not effective in certain animal IgGs, such as goat, sheep, cow, and horse. Protein G reacts with more IgGs than protein A and less reacts with other antibody types. However, protein G forms a weaker bond with antibodies than protein A. Thus, to compensate the weaknesses of both proteins A and G, genetic engineering on F_c-binding domains of proteins A and G has resulted in successful creation of a structurally and functionally chimeric protein (protein A/G) with broader binding than either protein A or G alone [15]. The protein A/G has two domains that can bind to the F_c domain of IgG; one domain is quite lysine-rich and a thiol-group can be incorporated into the lysine residue through reaction with 2-iminothiolane. When thiolated-protein A/G is immobilized onto a gold surface via self-assembly of gold and thiol group, the F_c-binding domains are exposed for binding with the F_c region of IgG without the loss of binding activity [16] (Fig. 1(b)).

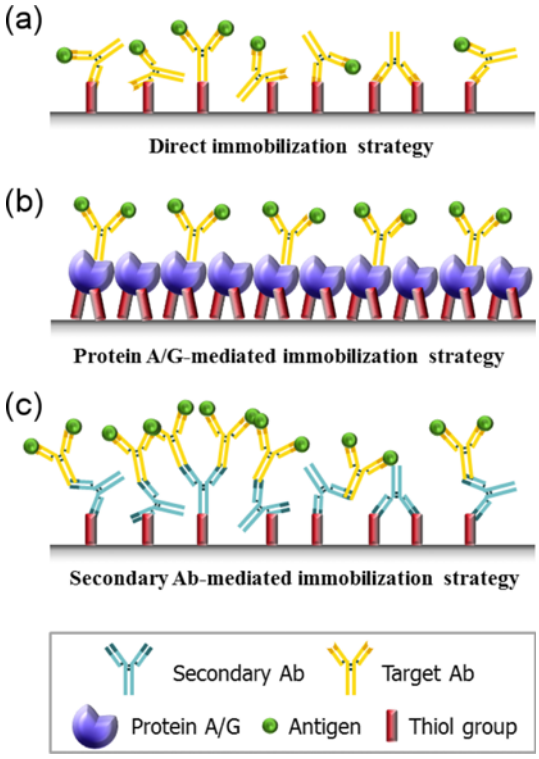

Fig. 1. Schematic representations of target antibody immobilizations on gold surface using (a) direct target antibody, (b) protein A/G-mediated, and (c) secondary antibody-mediated immobilization strategies.

Secondary antibody can recognize the F_c region of primary target antibody and it should match with the class or subclass of primary antibody. If the primary antibody is one of mouse IgG subclasses, any anti-mouse IgGs can be used as secondary antibodies. After immobilizing the thiolated-secondary antibody on gold surface, target antibody can be immobilized onto the layered secondary antibody by oriented binding between F_{ab} region of secondary antibody and F_c region of target antibody (Fig. 1(c)).

2. Comparative Analysis of Target Antibody Immobilizations using SPR

Target antibody immobilizations using direct thiol-conjugating, thiolated-protein A/G, and -secondary antibody were analyzed by SPR as a comparative tool. Although SPR result cannot show exactly

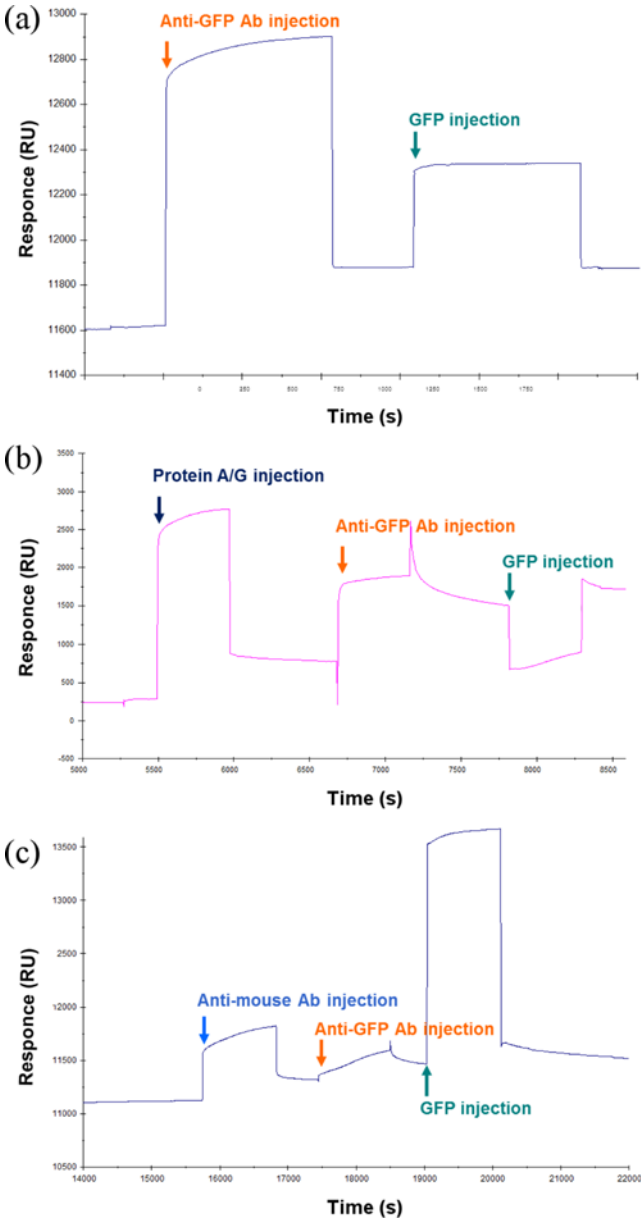

Fig. 2. SPR responses of anti-GFP antibody immobilizations and GFP antigen bindings using (a) direct target antibody, (b) protein A/G-mediated, and (c) secondary antibody-mediated immobilization strategies.

quantitative analysis, SPR is a good system for qualitative analysis. In the case of direct thiol-conjugation method for antibody immobilization, the RU value after injection of anti-GFP IgG increased suddenly from ~11,650 to ~12,700 (Fig. 2(a)). The RU value increased continuously during sample injection and finally reached ~12,900. After finishing injection and washing out non-specific binding, RU value dropped to ~11,900. Thus, net ~250 RU increase represented binding amount of target antibody. GFP antigen was injected after response was stabilized. Because GFP is relatively much smaller (~27 kDa) than anti-GFP IgG (~150 kDa), RU raise was smaller than that of antibody, showing net ~10 RU increase for antigen binding (Fig. 2(a)).

In the case of protein A/G-mediated method for antibody immobilization, thiolated-protein A/G and anti-GFP IgG were injected successively on gold surface in the same way, and ~255 RU and ~355 RU increases were determined, respectively (Fig. 2(b)). Different with protein A/G and anti-GFP IgG, GFP antigen showed going-down response profile at injection point due to different composition compared to electrolyte. Notably, GFP binding using the protein A/G-mediated immobilization strategy showed the highest response value (~105 RU). In the case of secondary antibody-mediated method as another tested oriented antibody immobilization strategy, thiolated-anti-mouse IgG and anti-GFP IgG were injected successively on gold surface, and ~190 RU and ~150 RU increases were determined, respectively (Fig. 2(c)). Although response of secondary antibody was relatively lower than the previous methods, immobilization amount of target antibody was somewhat high, which indicates that the use of secondary antibody can be one of the useful methods for antibody immobilization. For GFP antigen binding, net ~50 RU was increased (Fig. 2(c)).

3. Comparison of Antigen Bindings on Surface-immobilized Target Antibodies

Through RU changes in the SPR sensogram, degree of antibody immobilization could be simply determined as the following order: direct thiol-conjugated method>protein A/G-mediated method> secondary antibody-mediated method (Fig. 3(a)). However, antigen bindings on the antibody-immobilized surfaces resulted in different

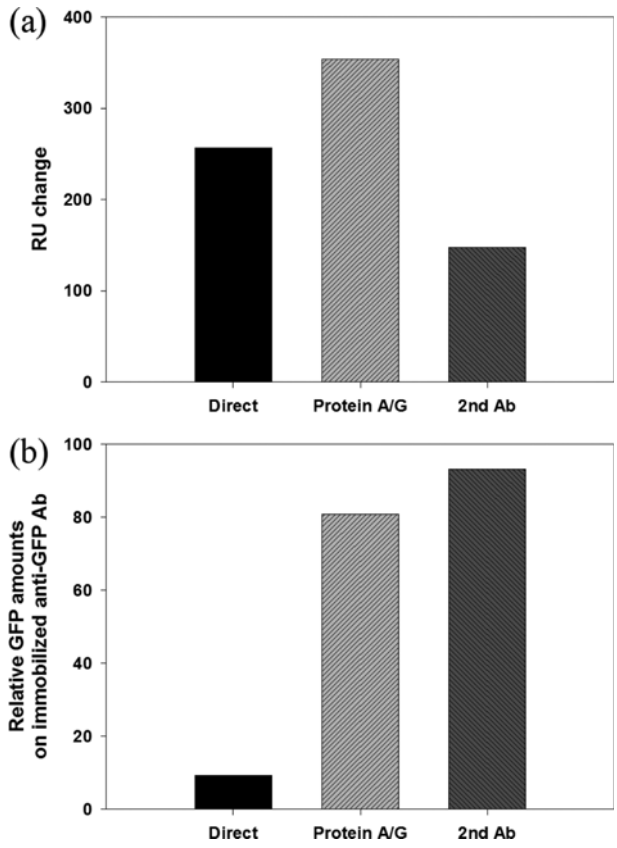

Fig. 3. (a) RU changes for anti-GFP antibody immobilizations and (b) relative GFP antigen amounts on immobilized anti-GFP antibodies using direct target antibody, protein A/G-mediated, and secondary antibody-mediated immobilization strategies.

order through simple RU changes: protein A/G-mediated method> secondary antibody-mediated method>direct thiol-conjugated method, which clearly indicates that oriented antibody immobilization caused efficient antigen binding.

Real-to-ideal ratio is an important criterion for oriented immobilization; a higher ratio indicates that more F_{ab} fragments are accessible to catch antigen molecules [17]. Thus, to analyze antigen binding on antibody-immobilized surface in more detail, a real-to-ideal ratio was considered. Antibody has two F_{ab} sites and molecular weight of GFP is ~5.56-times smaller than that of anti-GFP IgG. If all F_{ab} sites are fully exposed with activity after antibody immobilization, we can expect that ideal RU change of GFP is ~90 (=250×2÷5.56) based on ~250 RU change of anti-GFP IgG in direct thiol-conjugated immobilization method. Thus, we can surmise from comparison of ideal ~90 RU and experimental ~10 RU values for GFP antigen in direct thiol-conjugated method that F_{ab} sites were almost hidden and only ~10% F_{ab} sites were exposed outward. In the case of secondary antibody-mediated immobilization method, anti-GFP IgG was bound to ~80% of immobilized anti-mouse IgG. If all F_{ab} sites are fully exposed, ideal RU change of GFP would be ~54 (=150×2÷5.56) based on ~150 RU change of anti-GFP IgG. Thus, from comparison of ideal ~54 RU and experimental ~50 RU values, we calculated that GFP antigen bound to ~93% F_{ab} of anti-GFP IgG. Similarly, we determined that GFP antigen bound to ~80% F_{ab} of anti-GFP IgG in the case of protein A/G-mediated immobilization method. Collectively, by considering real-to-ideal ratio for analysis of SPR sensogram, we determined that total antigen binding levels have the following order (Fig. 3(b)): secondary antibody-mediated method>protein A/G-mediated method>direct thiol-conjugated method.

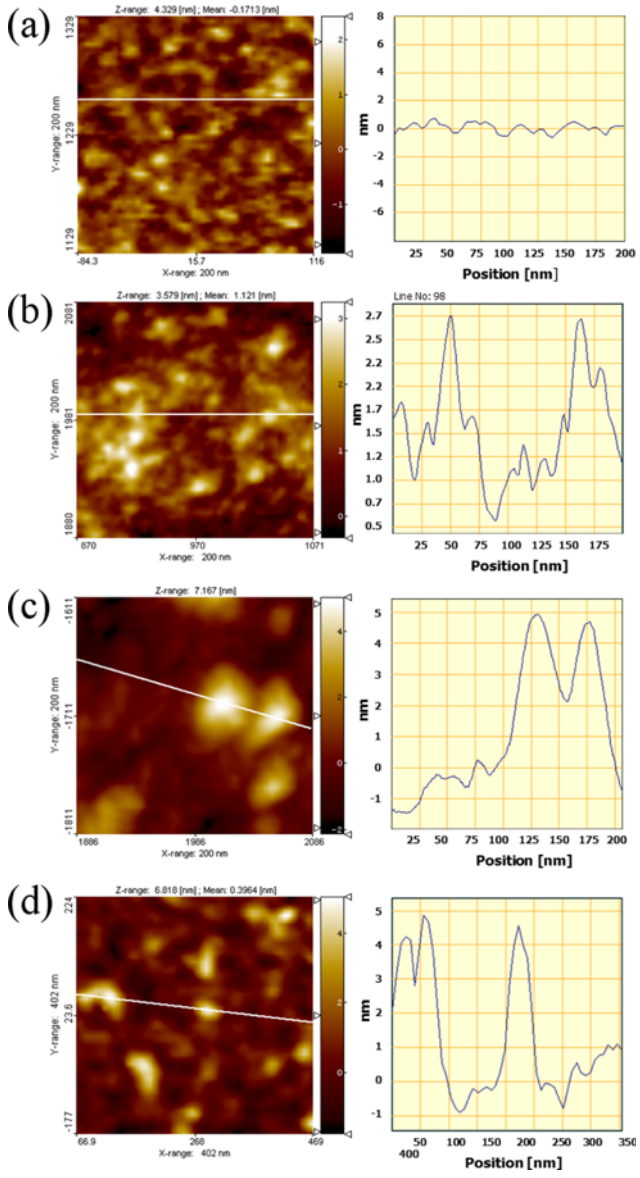

Fig. 4. AFM images of (a) bare gold surface and anti-GFP antibody-immobilized gold surfaces using (b) direct target antibody, (c) protein A/G-mediated, and (d) secondary antibody-mediated immobilization strategies.

It is clear that the use of protein A/G is the most efficient strategy in terms of antibody immobilization, which can be judged from the highest RU value of the SPR sensorgram. However, when considering antigen binding capacity with real-to-ideal ratio, we can conclude that secondary antibody-mediated method would be a promising oriented antibody immobilization strategy.

4. Surface Topography Analysis Using AFM

There are several scanning probe microscopic studies involving imaging of antibody on surface in a nanometer scale [18,19]. We performed AFM analysis for topographical images of different immobilizations. Through analysis of bare gold chip as negative control, average height was shown under 1 nm (Fig. 4(a)). When anti-GFP IgG molecules were bound on gold surface using direct thiol-conjugating method, we observed some bright spots having high height which might have head-on or head-end orientation on surface and major spots having low height which might be due to side-on orientation (Fig. 4(b)). Anti-GFP IgG molecules which bound on protein A/G-immobilized surface had bright spots having high height about 4-5 nm (Fig. 4(c)). Similarly, anti-GFP IgGs were also bound onto anti-mouse IgG-immobilized surface, showing highly bright spots (Fig. 4(d)). These spots might be combined molecules of anti-mouse and anti-GFP IgGs, leading to an increase in surface height. Dark spots in background might be non-coupled anti-mouse IgGs with non-oriented binding.

CONCLUSIONS

We compared three antibody immobilization methods for convenient and effective applications: direct thiol-conjugated immobilization as control and thiolated-protein A/G-mediated and thiolated-secondary antibody-mediated methods as oriented immobilizations. GFP was used as model target antigen and anti-GFP IgG as target antibody. Real-to-ideal ratio for antigen binding, which is an important criterion for oriented immobilization, was considered for analysis of SPR response. Even though, total immobilization levels of target antibody had following order--direct thiol-conjugated immobilization>protein A/G-mediated immobilization>secondary antibody-mediated immobilization--oriented immobilization levels of target antibody (i.e., total antigen binding levels) showed the following order: secondary antibody-mediated immobilization>protein A/G-mediated immobilization>direct thiol-conjugated immobilization. AFM topographical analyses of three immobilization meth-

ods might support these explanations. We confirmed that protein A/G- and secondary antibody-mediated strategies which consider orientation of target antibody immobilization showed significantly higher antigen binding efficiencies than that of direct thiol-conjugated strategy. Notably, secondary antibody-mediated immobilization strategy showed the best target antigen binding efficiency based on same target antibody amount. Thus, the oriented antibody immobilization methods using linker materials such as protein A/G and secondary antibody could be useful in diverse antibody-antigen interaction-involved application fields including immunoassays, biosensors, and affinity chromatography.

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