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# Construction of His6-Protein A/PEG Co-Immobilized Surface for High-Performance Protein Sensing

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Development of the biointerface for high performance immuno-sensing methods is one of the current research topics in analytical chemistry and biochemistry. We developed histidine-tagged protein A (His6-protein A)/poly(ethylene glycol) (PEG) co-immobilized gold surface as a new class of immuno-sensor chip, where both protein A and PEG are directly immobilized on the surface by the histidine-tag and the thiol group, respectively. In this study, the resulting surface was applied to sandwich assay for specific protein detection based on surface plasmon resonance measurements. Despite the similar content of immobilized antibodies, the amount of secondary antibody on His6-protein A/PEG surface was about eight times higher than that of physically adsorbed antibody/PEG surface. In addition, nonspecific protein absorption was extremely suppressed on His6-protein A/PEG surface, compared with $Ni^{2+}$–nitrilotriacetic acid (NTA) surface, which was generally used for the immobilization of histidine-tagged proteins. These results clearly indicate that effective orientation of the immobilized antibody with the prevention of non-specific adsorption was achieved on the His6-protein A/PEG surface.

Key words: SPR, His6-protein A, PEG brush, Antibody, Sandwich assay, High sensitive detection

## INTRODUCTION

Antibody arrays have considerable potential in a variety of applications including proteomics research, drug discovery, and immuno-diagnostics. It is important to fabricate the antibody arrays at a uniform density and in an aligned orientation with the prevention of non-specific adsorption.

Several methods have been proposed for preparing oriented protein molecules on solid-surfaces described as follows: the DNA-antibody conjugates, which were prepared via a covalently connection between a thiolated-end of single stranded DNA and the antibody by sulfosuccinimidyl 4-(*p*-maleimidophenyl) butyrate (sulfo-SMPB)[1]. These DNA-antibody conjugates were immobilized directly to the complementary ssDNAs on the gold surface and specifically convert the DNA chip into a protein chip. Fab′-SH fragments can also be immobilized on gold surface via Au-S bond using free thiol group in Fab′-SH[2,3]. Fab′-SH fragments were obtained from the controlled chemical reduction of $F(ab')_2$, which was a truncated form of whole molecule IgG. This step cleaves the single disulfide bond, which connects the two heavy chains of $F(ab')_2$ together, to form two Fab′-SH fragments. The following is recent trend in the oriented immobilization of proteins. Histidine-tagged protein A bound to $Ni^{2+}$-NTA surface[4], which was generally used for the immobilization of histidine-tagged proteins. These studies demonstrated that the engineered protein exhibited high affinity toward antigen when binding site of protein was selectively immobilized on surface.

In order to improve protein specific interactions on the biosensor surface, not only high sensitivity but also low background signals are required. Nonspecific adsorption of biocomponent is one of the most severe problems because a biofluidic sample contains numerous biomolecules such as proteins and lipids. Thus, to prevent nonspecific adsorption, a variety of modifications on the surface has been carried out. Though carboxymethyldextran surfaces[5] was generally used as a surface plasmon resonance (SPR) sensor surface, it is difficult to avoid the nonspecific adsorption completely based on the electrostatic interaction. Self assembled monolayer (SAM)[6] of small molecular weight thiol compound such as 1-mercapt undecanol is reported to reduce nonspecific adsorption. Nevertheless, the performance of this method is not good enough because the construction of SAM needs time-consuming work and organic solvents in addition to the limited non-fouling character. Though the additional approaches such as an immobilization of oligo(ethylene glycol) on the SAM surface have been done, the performance

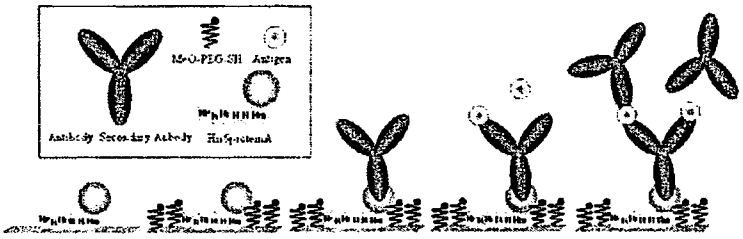

Figure 1. Schematic (not to scale) illustration of a sandwich assay using His6-protein A/PEG co-immobilized gold surface.

has not been satisfied enough. Poly(ethylene glycol) (PEG) is known as a useful blocking agent for biomaterials surface. PEG has unique properties such as solubility and flexibility of the chains and basicity of the ether oxygens in the main chain[7,8]. One of the most important utilizations of PEG is the construction of polymer brushes[9], a densely packed layer of tethered polymers anchored on the surface utilizing one of the end functionality of the polymer chain. Because that PEG is electrically neutral[10], PEGylated surface can also avoid the nonspecific adsorption based on electrostatic interaction. So far, we have synthesized several types of heterotelechelic PEGs. For example, a heterobifunctional PEG possessing an acetal group at one end and a mercapto group at the other end (α-acetal-ω-mercapto-PEG) on the sensor chip appreciably reduced the nonspecific adsorption of bovine serum albumin (BSA)[11]. Heterotelechelic PEG tethered chain is feasible to immobilize protein at a distal end of PEG chain. Uchida et al. carried out a biotinylation of the aldehyde free end of the tethered PEG which was liberated by the acid hydrolysis of the acetal end group, followed by a reaction with biocytin hydrazide under a reduced conditions. In this method, it was not easy to control orientation for immobilization of protein and often difficult to increase the amount of immobilized protein.

Several reports on the immobilization of antibody in a highly oriented manner with prevention of non-specific adsorption are available. In order to improve the orientation of antibodies on substrate surface, protein A which exhibits a specific interaction with the Fc chain of immunoglobulin G (IgG) can be used. Our idea was to effectively immobilize protein A in a oriented fashion utilized protein A having oligoamine. Note that the PEGylated gold-nanoparticles formed from PEG-poly[2-(N,N-dimethylamino)ethyl methacrylate] (PAMA) block copolymer showed excellent stability under physiological conditions even in the presence of high concentrations of thiol compounds, due to the multivalent coordination in addition to the electrostatic interaction between the negatively charged gold surface and the tertiary amino groups of the PAMA segment[12], suggesting that the surface composed of mercapto-ended PEG (SH-PEG) and protein A possessing oligoamine chain may construct the smart PEGylated gold sensor surface. In this study, we engineered a hexahistidine tag at the C-terminus of protein A (His6-protein A). Based on the interaction between the gold surface and the amino groups of oligohistidine adjacent to the C-terminal of the protein A, an effective orientation of immobilized protein is anticipated. PEG was utilized as a blocking reagent after the immobilization of His6-protein A to improve the non-fouling character. This paper describes a strategy to immobilize antibodies in a controlled orientation using the interaction between oligoamine and gold surface.

## EXPERIMENTAL SECTION

**Materials.** A gold chip (SIA KIT Au) for SPR measurement was purchased from Biacore AB (Uppsala, Sweden). BSA was purchased from Sigma-Aldrich Fine Chemicals. IgG fraction rabbit anti-bovine albumin (anti-BSA) was obtained from Inter-Cell Technoloies, INC. MeO-PEG-SH (M.W. 2000) was purchased from NOF Corporation. A solution of 0.01 M HEPES buffer (pH 7.4, containing 0.15 M sodium chloride, 3 mM EDTA, and 0.005% surfactant P20) was purchased from Biacore AB (Uppsala, Sweden). His6-protein A was prepared according to a litrature[13]. SPR evaluations were carried out on a Biacore 3000 device (Biacore AB). IODINE-125($^{125}$I) was purchased from Amersham Biosceinces. Sodium N-chloro-p- toluenesulfonamide (chloramine T) was purchased from Wako.

**Construction of sensor chips.** Figure 1 shows the schematic (not to scale) illustration of a procedure for the construction of His6-protein A/PEG co-immobilized surface (His6-protein A/PEG surface). Immobilization of His6-protein A and PEG-SH (2k) on a gold surface were carried out on sensor chip in SPR instrument at 25 °C. Briefly, gold surfaces were cleaned with piranha etching solution (3:1 concentrated $H_2SO_4/H_2O_2$) at room temperature and then rinsed with copious amounts of water prior to use. The cleaned sensor chip was docked into the instrument and 1 μM His6-protein A in a solution of 0.01 M HEPES buffer was injected at constant flow rate of 5 μl/min for 30 min. After immobilization of His6-protein A was done, 0.1 mg/ml of PEG-SH (2k) solution was injected at constant flow rate of 5 μl/min for 30 min to fill the un-modified area of gold substrate.

Similarly, construction of the anti-BSA/PEG co-immobilized surface (Anti-BSA/PEG surface) was carried out in SPR instrument at 25 °C. 1 μM anti-BSA antibody in 0.01 M HEPES buffer solution was injected (5 μl/min, 30 min) to physically adsorbed antibody on gold sensor surface. Subsequently, 0.1 mg/ml of PEG-SH (2k) solution was injected (5 μl/min, 30 min).

Table 1. Surface coverage of immobilized protein bare gold surface.

| protein | SPR angle shift (°) | amounts of pro (ng/nm$^2$) | occupation area per molecule (nm$^2$)[a] | surface coverage (%) |
|---|---|---|---|---|
| Anti-BSA | 0.13±0.05 | 0.77±0.30 | 120 | 37±16 |
| Protein A | 0.04±0.04 | 0.24±0.24 | 64.5 | 22±22 |
| His6-proteinA | 0.17±0.04 | 1.01±0.24 | 64.5 | 93±22 |

[a]Occupation area per molecule was estimated using assumption as lows; side-on immobilization, retention of the structure of protein immobilized on the surface, and constructed protein-layer wa onolayer.

Ni-NTA-His6-protein A surface, which means that His6-protein A was immobilized on $Ni^{2+}$–NTA surface, was constructed according to the literature[14, 15, 16]. The gold sensor chip was immersed in 50 mM phosphate buffer (pH 7.4) containing 100 μM 3,3'-dithiobis [*N*-(5-amino-5-carboxypentyl) propioamide-*N*',*N*'-diacetic acid] dihydrochloride for overnight at room temperature to form a SAM terminated with NTA groups on the surface. Subsequently, the gold sensor chip was dipped in 40 mM $NiSO_4$ aqueous solution for a certain period to bind nickel ions to the NTA groups. After the $Ni^{2+}$–NTA chip was docked into SPR instrument, immobilization of His6-protein A was carried out in a similar way to stated above on sensor chip.

**Surface coverage of His6-protein A on a gold surface.** The surface coverage of proteins on the gold surface was determined by radioisotope labeling method. Antigen-binding fragments of antibodies ($F(ab')_2$) were radiolabeled with $^{125}I$ using chloramine T as an oxidizing agent. 200 μl of the protein solution (1 mg/ml) was mixed with 20 μl of carrir-free $^{125}I$ and 90 μl of chloramine T solution (3 mg/ml) and the mixture was allowed to react at room temperature for 1 min. The reaction was quenched by adding 100 μl sodium metabisufite solution (10 mg/ml) for 2-3 min. Unreacted iodine was removed by gel permeation chromatography. Radiolabeled samples were counted using a Beckman radio isotope detector. After the radiolabeled $F(ab')_2$ fragment was reduced to Fab' fragments by dithiothreitol, imobilization was carried out using the SPR. By using the immobilization data, it was confirmed that 0.5937 ng/mm$^2$ of antibody was equivalent to 0.1 ° of SPR angle shift. Surface coverage of protein A and His6-protein A on a gold surface were determined by the SPR data assuming the size of protein A as length of 25 nm, width of 2.588 nm[17, 18] and molecular weight of 42,000. Similarly, surface coverage of anti-BSA antibody was estimated using the known values; length of 12 nm, width of 10 nm and molecular weight of 150,000.

**Recognition of protein on the constructed sensor chips.** Detection of target antigen (BSA) was carried out using SPR instrument based on a sandwich assay described as follows; 1 μM anti-BSA antibody in 0.01 M HEPES buffer solution was injected at a constant flow rate of 5 μl/min for 30 min to immobilize the antibody on the constructed sensor chips. Various concentration of antigen (BSA) in 0.01 M HEPES buffer solution was then injected at a constant flow rate of 5 μl/min for 30 min. 1 μM secondary antibody (anti-bovine serum albumin antibody) was injected to the antigen contacted surface at a constant flow rate of 5 μl/min for 30 min for a sandwich assay. All experiments were carried out at 25 °C and constant flow rate of 5 μl/min.

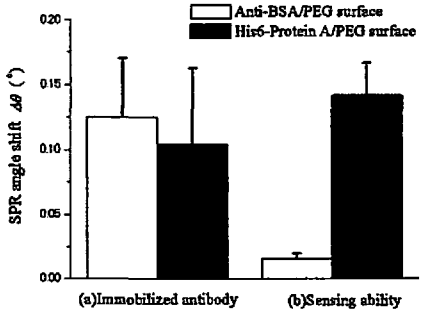


Figure 2. SPR angle shifts of the antibody immobilization (a) and sandwich assays of 100 nM antigen (b) on the constructed surfaces.

## RESULTS AND DISCUSSION

Prior to the analysis of specific binding between protein samples and the corresponding antibody on the surface, the effectiveness of oligohistidine at the end of protein A chain toward gold surface was investigated. As shown in Table 1, the immobilized amounts of physically absorbed anti-BSA and protein A on a gold surface were determined to be 0.74 ng/nm$^2$ and 0.24 ng/nm$^2$, respectively. In contrast, the immobilized amount of His6-proteinA was 1.01 ng/nm$^2$. From these results, the surface coverage of His6-protein A was estimated to 93%, while that of physically adsorbed proteins were less than 40% (Table 1). These results suggested that His6-protein A was immobilized to a gold surface efficiently via specific binding by oligoamine (His6) chain.

PEG modification was carried out on a sensor surface reduce a nonspecific adsorption of biomolecules. Thus protein/PEG co-immobilized gold surface can be easily constructed by the consecutive treatments with the protein and the PEG-SH solutions. To evaluate the further functions of His6 chain at the end of protein A, the amount of the immobilized antibody on the His6-protein A/PEG surface was compared with the other control surfaces. The amounts of immobilized antibody on the His6-proteinA/PEG surface are seven times higher than that on protein A/PEG surface, where the native protein A, which is physically adsorbed on a gold surface, with PEG (data not shown). This result

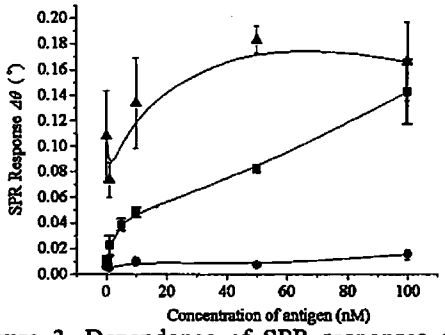


Figure 3. Dependence of SPR responses on the concentration of antigen. (▲) Ni-NTA His6-protein A surface, (■) His6-protein A/PEG surface, and (●)Anti-BSA/PEG surface. Detection of the antigen was carried out by SPR instrument based on sandwich assay using 1 μM secondary antibody.

suggested that His6 chain works as a linker to improve its amount on the surface, which may cause improve in aligned antibody on the surface. Though the amounts of physically immobilized antibody on the gold surface (0.77 ng/nm$^2$) and specifically immobilized antibody on the His6-protein A/PEG surface (0.62 ng/nm$^2$) were almost the same (Figure 2a), however, the SPR response of sandwich assay on His6-protein A/PEG surface was about eight times higher than that of physically adsorbed antibody/PEG surface (Figure 2b), indicating that the effective orientation of the immobilized antibody was achieved on the His6-protein A/PEG surface.

Figure 3 shows the results of sandwich assays of the constructed surface based on SPR measurements. As a control, we used Anti-BSA/PEG surface and Ni-NTA-His6-protein A surfaces. Since the immobilized amount of the antibody on the Ni-NTA-His6-protein A surfaces was higher than that on the other surfaces, largest SPR angle shift was observed when sandwich assay was performed on Ni-NTA-His6-protein A surface (Figure 3). However, non-specific adsorption of secondary antibody was extremely high in the case of Ni-NTA-His6-protein A surface. On the contrary, is PEG brush effectively worked on the surface to reduce nonspecific adsorption in the case of the Anti-BSA/PEG surface and His6-protein A/PEG surfaces. In consideration with the high specific signal of His6-protein a/PEG surface, it is confirmed that the highest sensitivity was achieved by the His6-protein A/PEG surface.

## CONCLUSIONS

Protein A carrying His6 chain was found to immobilize on gold surface effectively. The immobilized protein A worked to capture antibody on the surface, indicating that the effective functionality of the His6-protein A/PEG surface, *viz.*, the immobilization of antibody in a highly oriented manner with prevention of non-specific adsorption. This His6-protein A/PEG co-immobilized surface achieved specific biosensing with higher S/N ratio than Ni-NTA-His6-protein A surface, which is commonly used as a protein-immobilization substrate. These results represent significant advances toward the expansion of SPR biosensing to a wider range of biochemical interactions.

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