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Strategies in Protein Immobilization on a Gold Surface

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Protein immobilization on a gold surface plays an important role in the usefulness of biosensors that utilize gold-coated surfaces such as surface plasmon resonance (SPR), quartz crystal microbalance (QCM), etc. For developing high performance biosensors, it is necessarily required that immobilized proteins must remain biologically active. Loss of protein activity and maintenance of its stability on transducer surfaces is directly associated with the choice of immobilization methods, affecting protein-protein interactions. During the past decade, a variety of strategies have been extensively developed for the effective immobilization of proteins in terms of the orientation, density, and stability of immobilized proteins on analytical devices operating on different principles. In this review, recent advances and novel strategies in protein immobilization technologies developed for biosensors are briefly discussed, thereby providing an useful information for the selection of appropriate immobilization approach.

Keywords : Protein immobilization, Biosensor, Gold surface, Orientation, Immobilization technique

I. Introduction

A biosensor, which is capable of converting biological responses into measurable signals, typically consists of three main components such as a transducer, a target analyte, and capture agents (i.e., protein, antibody, enzyme, peptide, DNA, etc) [1]. The selective immobilization of proteins as a bio-recognition process is a key step in developing valuable biosensors, because both protein stability and functionality require special control over protein orientation at the thin layers of devices [2,3]. So far, a variety of methods have been developed for protein immobilization in biosensor applications, as the orientation-controlled immobilization of proteins is crucial for the sensitive and accurate measurements. There are some common aspects on protein immobilization processes highly associated with a sensing performance, which include denaturation or conformational changes in proteins after immobilization, thereby leading to decrease in protein detection sensitivity [4]. In general, the non-specifically adsorbed proteins to surfaces are largely responsible for this kind of undesired insensitiveness. The oriented immobilization of proteins has become a critical issue for characterization as well as for detection of biomolecular interactions in biosensors, because biological function of proteins at the solid supports is determined by their unique

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conformation. Evenly oriented immobilization also provides high consistency and reproducibility for the fabrication of stable protein biosensors. Thus, a common pivotal step for the development of most protein biochip is the efficient immobilization of captured proteins on the solid supports of assay devices. Commonly used solid supports include gold, silicon, glass, plastic, etc., and more recently, various nanostructured materials such as nanoparticles, nanowires, and nanotubes [5].

It is obvious that protein chip technology has drawn much attention in the field of proteomics due to its applicability for simultaneous and high-throughput screening study [6]. So far, many label-free technologies combined with protein biochip have been developed. Among these technologies available, a bio-analytical system that has received huge attention involves surface plasmon resonance (SPR), which enables us to directly detect biomolecular interactions. SPR technique is capable of detecting changes in refractive index (n) occurring near the thin metal layers (i.e., gold films) within ~200 nm. The changes in the SPR angle can be measured by recording the intensity of the reflected light with the angle of incidence [7]. Remarkable developments can be seen in the field of SPR biosensors, covering wide range of applications in biomedical, biochemical, and environmental areas, as a SPR spectroscopy also provides real-time monitoring of molecular binding [8,9]. Just like other types of biosensors, non-specific adsorption (NSA) of protein to surfaces is the biggest drawback in SPR biosensor applications. Along this line, it is thought that major problems in the immobilization process of functional proteins to solid supports are instability of bound proteins and loss of their binding activity caused by the randomly oriented molecules on the surface. Here, we will discuss recent development of protein immobilization methods in SPR-type biosensors in terms of the orientation, stability, and density of immobilized proteins to gold surface.

II. Strategies of Protein Immobilization

1. Bio-affinity immobilization

By virtue of advances in molecular biology techniques, the use of a genetically fused affinity fusion partner enabled us to purify the target proteins, as affinity tag can be fused to the protein of interest [10]. Affinity chromatography based on fusion partner-specific affinity makes use of purification of molecules of interest. In addition to the protein purification, affinity fusion tags are also applicable for detection of expressed protein as well as immobilization of proteins to solid supports on the basis of bio-affinity binding. Fig. 1 shows a simplified schematic representation of on-chip detection of GST-tagged and His6-tagged recombinant proteins on glutathione- and Ni-NTA-coated layers.

Through the engineering of target protein with specific affinity tags, the rapid monitoring of recombinant proteins based on SPR imaging system

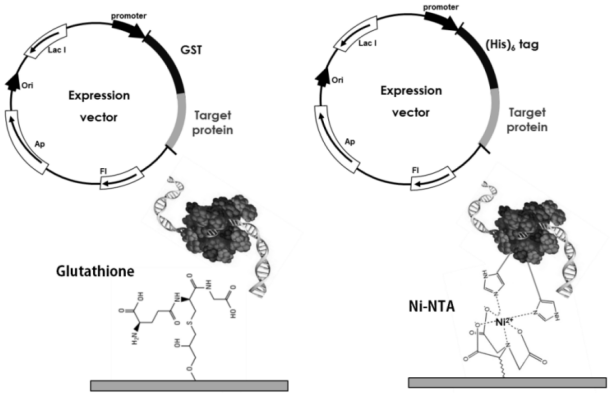

Figure 1. Physical map of the plasmid for expression of recombinant fusion proteins. Target proteins genetically fused with glutathione S-transferase (GST)-tag (left panel) and hexahistidine (His6)-tag (right panel) interact with glutathione- and Ni-NTA-modified surfaces, respectively.

has been performed by Jung et al. [11]. The authors described the usefulness of SPR imaging (SPRI) system to detect the expression of affinity-tagged proteins such as hexahistidine (His6)-, glutathione S-transferase (GST)-, and maltose binding protein (MBP)-fused proteins. In their study, the hexahistidine/ubiquitin-tagged human growth hormone (His6:Ub:hGH), glutathione S-transferase-tagged human interleukin 6 (GST:hIL6), and maltose binding protein-tagged human interleukin 6 (MBP:hIL6) were expressed in *Escherichia coli*., and then analyzed using SPRI. For convenient orientation-controlled immobilization of antibody, genetic fusion approaches to protein G based on bio-affinity tags have been carried out [12-15]. Antibody-binding proteins have been genetically engineered with various fusion tags such as glutathione S-transferase [12], hexahistidine [13], elastin-like protein [14], and oligonucleotides [15]. Elastin-like protein-fused antibody-binding proteins have been used for temperature-triggered purification of antibody purification as well as for the fabrication of antibody arrays on the glass slide [14]. GST- or His6-fused protein G exhibited well-oriented immobilization on glutathione- or Ni-NTA-coated surfaces, respectively. The hexahistidine (His6) tag is the most commonly used bio-affinity tag for purification, detection and immobilization of recombinant proteins in biology due to its small size, thus rarely affecting the functional properties of tagged proteins [11,16]. Recently, it has been reported that histidine tag can bind to a gold surface [17]. What this means is that the fused molecules with histidine tag may adsorb nonspecifically to the gold surface. Thus, the nonspecific adsorption of tags of six histidine residues on direct immobilization of target protein to the gold thin film remains to be elucidated.

2. Cysteine-mediated immobilization

A large number of studies have been carried out on the improvement of protein immobilization with orientation, since orientation-controlled immobilization is relevant in creating more selective and sensitive biosensors [18,19]. Well-oriented immobilization of proteins to a gold surface commonly occurs in the presence of functional groups such as thiol (-SH) that possesses a strong affinity for gold through forming gold-sulfur interaction. Recently, numerous studies have reported thiol-specific cross-linking and site-specific cross-linking of sulfhydryl group to a gold surface [20,21]. It is well-known that thiol adsorption occurs on the gold surface. So, sulfur-containing molecules can bind to gold. One example of the self-oriented immobilization is the cysteine-mediated immobilization [22]. By virtue of genetic engineering technique, cysteine amino acids can be introduced at a specific site in proteins. Thus, direct immobilization of proteins attached by cysteines at gold surfaces has been beneficial to biosensing applications.

3. Protein G-mediated immobilization

Studies on direct immobilization of antibody on a solid support using protein G, which is an antibody binding protein expressed in Streptococcal bacteria, have been extensively performed [15,22]. Protein G uniquely binds to the Fc portion of an antibody, providing the proper orientation of tightly bound antibody on solid supports. Thus, antigen binding sites of antibody will be exposed upward away from the surface, and antigen binding activity remains unaffected accordingly [23,24]. Protein G-mediated antibody immobilization strategy usually does not need any step of antibody pre-modifications, so immobilized antibodies do not lose their binding specificity and affinity. Thus, this approach offers some benefits such as high degree of sensitivity, reduced overall time required for preparation of functional surfaces, and cost-effectiveness over

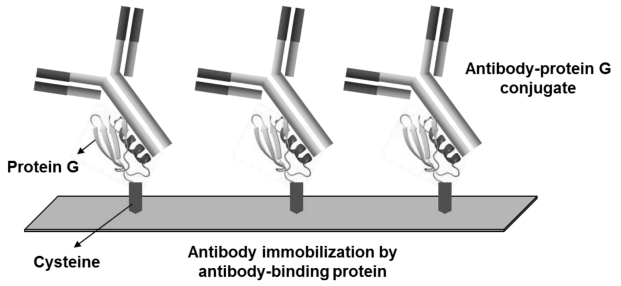

Figure 2. Strategy of antibody immobilization onto gold surface based on antibody-binding protein-mediated immobilization [22].

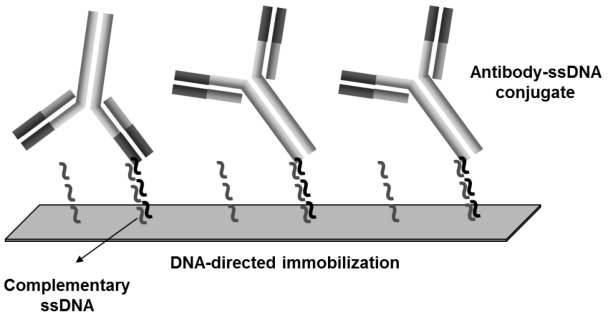

Figure 3. Strategy of antibody immobilization onto gold surface based on DNA-directed immobilization [30].

conventional immobilization techniques (i.e., biotin-streptavidin interaction, covalent coupling, etc.). Immunoassay methods based on the use of antibody immobilized via protein G usually display enhanced sensing abilities compared to traditional random covalent immobilization techniques.

Genetically engineered recombinant form of protein G can be produced in large quantities using recombinant DNA technology that provides the possibility to generate fusion proteins. Protein G is amenable to genetic and chemical modification for orientation-controlled immobilization of antibody. As shown in Fig. 2, the use of protein G genetically fused with cysteine residues allowed for well-ordered orientation, and remarkably improved the subsequent antigen binding to bound antibody [22]. In that study, the antibody density on cysteine-introduced protein G surface was found to be around four times higher than that on a randomly oriented protein G surface. Thus, it is convincingly thought that oriented immobilization of antibodies on protein G monolayers is appropriate for antigen binding. Moreover, strategy for oriented immobilization using protein G can be also applied to other antibody-binding proteins (i.e., protein A, protein L, protein A/G, etc.) to broaden the span of possible antibodies. Although protein G-mediated immobilization method has been considered to be useful and effective for various types of detection platforms, there appears to be a small issue that this technique needs additional attachment of protein G onto the surface prior to the immobilization of antibody.

4. DNA-directed immobilization

DNA-directed immobilization of proteins is an efficient method for generating well-oriented pattern of proteins on a DNA-functionalized surface by assembling target proteins on a DNA surface [25]. DNA surface is heat-stable, robust and inexpensive, and also relatively easy to develop in comparison with protein surface. DNA-directed protein immobilization is a fast oriented immobilization method, thereby allowing us to avoid long incubation times under harsh condition at the thin layers of devices.

Some strategies allowing controlled immobilization of antibody on a DNA surface have been reported [26-28]. One example of these strategies is the use of streptavidin-DNA conjugates [29]. Through the interaction between biotin and its ligand, streptavidin, biotinylated antibodies could be immobilized on the streptavidin-DNA-patterned surface in orientation-controlled manner. As shown in Fig. 3, antibodies could also be conjugated to DNA and directly addressed to DNA-functionalized gold layers [30]. However, in order to make these methods useable, antibodies as capture agents must be covalently modified

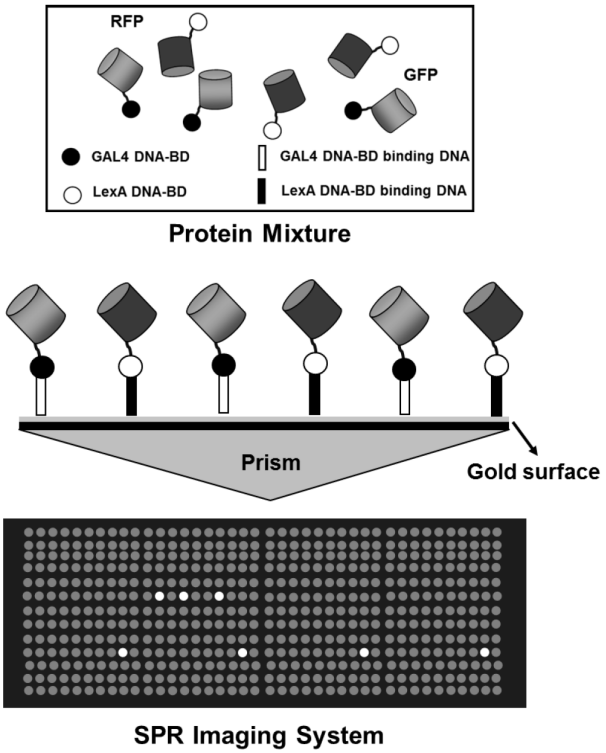

Figure 4. Schematic representation of site-directed immobilization of DNA-binding proteins on DNA-patterned gold chip surfaces [31]. This method is primarily based on the specific interaction between GAL4 DNA-BD and upstream activating sequence (UAS), and between LexA DNA-BD and LexA operator (LexA op). GFP, green fluorescent protein; RFP, red fluorescent protein.

with DNA or small molecule (i.e., streptavidin), which may cause chemical modifications at the antigen binding sites, leading to the blockade of the antibody ability to bind antigen. Likewise, study on protein-DNA conjugates has been performed by Boozer et al., reporting a stable and versatile biosensor surface based on site-directed immobilization of protein-DNA conjugates onto a mixed self-assembled monolayer (SAM) [26].

Approach of DNA-specific immobilization is also applicable for the development of protein function array systems [31]. Protein biomolecules are not the only capture agents for protein function array. Alternative reagent includes nucleotides, which can be a special agent for protein capture, to be more exact, DNA binding protein. A large number of interactions of proteins on DNA-modified layers have been studied by the use of SPR-type biosensor systems [32,33], which include the interactions between the cMyb DNA-binding domain and its sequence-specific cis-acting element [34], and the binding of p53 tumor suppressor to it cognate DNA sequences [35].

Jeong et al., reported an effective technology for the site-directed immobilization of proteins with DNA-binding domains onto cognate DNA surfaces [31]. The authors utilized the well-characterized DNA-binding domains derived from two different transcription factors, the yeast GAL4 [36] and bacterial LexA [37], to immobilize target proteins to DNA-modified layers (Fig. 4). Target proteins, GFP (green fluorescent protein) and RFP (red fluorescent protein), were fused with two different DNA-binding domains, GAL4 DNA-BD and LexA DNA-BD, respectively. The resultant DNA-binding fusion proteins, GAL4 DNA-BD:GFP and LexA DNA-BD:RFP, were addressed to the DNA-functionalized gold thin film, and subsequently analyzed by SPR imaging measurement. The sequence-specific site-directed immobilization is highly usable not only for investigating the DNA binding profiling of transcription factors, but also for analyzing the protein-protein interactions between DNA-binding proteins and their binding partners on the DNA surfaces.

5. Orientation-controlled high density immobilization

Besides of benefit of the orientation-controlled immobilization of proteins, packing density, which is directly associated with accessibility of the immobilized molecules on a sensing layer, can have a significant effect on the sensitivity and efficiency of biosensors, allowing for measurements at lower concentration of

an analyte. The formation of oligomeric protein is mainly mediated by the involvement of oligomerization domain, which is responsible for the functional properties of the protein, typically by strengthening interaction, or enhancing structural stability. This biochemical properties of protein assembly can be mimicked through molecular biology technology by combining the target protein gene with the gene corresponding to the oligomerization domain.

Recently, Park et al., employed a surface plasmon resonance (SPR) to immobilize target proteins with high density in orientation-controlled way [38]. The target protein (EGFP) was modified with two cysteines and oligomerization domains for orientation-controlled high density immobilization (Fig. 5). To construct oligomeric EGFP proteins with two cysteines at the C-terminal region, the leucine zipper domain of the yeast transcription factor GCN4, which is one of the best characterized and the most frequently used oligomerization domain (OD), was utilized for the effective assembly of the oligomerization states of the biomolecule. The GCN4 leucine zipper consists of 33 amimo acids that form a two-stranded parallel coiled-coil of helices [39]. Mutations in a- and d-potisions of leucine zipper cause abnormal oligomers to form. Thus, the use of mutagenesis approach enables us to produce the variants (or mutants) of wild type dimeric GCN4 leucine zipper, thereby displaying various oligomeric states [40]. In order to obtain oligomeric EGFP:Cys proteins, dimeric and trimeric segments derived from native GCN4 leucine zipper were genetically combined with EGFP protein that provides direct visualization of its adsorption. DNA sequences and the encoded amino acid sequences of wild type GCN4 leucine zipper (dimerization domain) and mutant form (trimerization domain) are shown in Table 1.

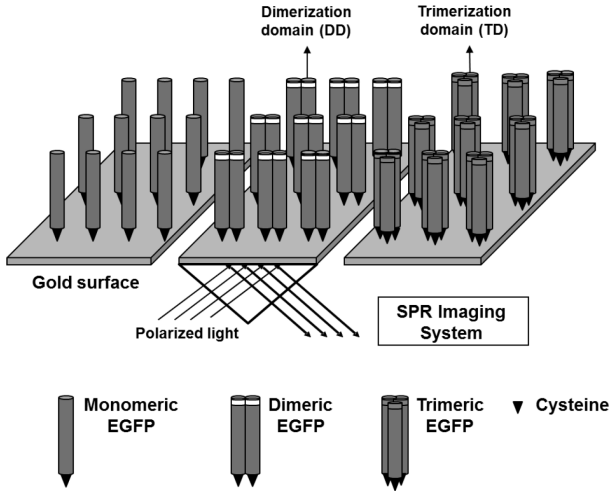

Figure 5. Schematic representation of the forms of the oligomeric EGFP:Cys chimeric proteins for orientation-controlled high density immobilization [38].

After treatment with monomeric EGFP:Cys proteins at a gold substrate, the amount of bound protein was

Table 1. Nucleotide sequences and the encoded amino acid sequences in proteins of GCN4-derived dimerization domain and trimerization domain. Symbols enclosed in parentheses denotes any amino acid [38].

Domain	Nucleotide and amino acid sequences							
Dimerization	AGA(R)	ATG(M)	AAA(K)	CAA(Q)	CTT(L)	GAA(E)	GAC(D)	AAG(K)
	GTT(V)	GAA(E)	GAA(E)	TTG(L)	CTT(L)	TCG(S)	AAA(K)	AAT(N)
	TAT(Y)	CAC(H)	TTG(L)	GAA(E)	AAT(N)	GAG(E)	GTT(V)	GCC(A)
	AGA(R)	TTA(L)	AAG(K)	AAA(K)	TTA(L)	GTT(V)	GGC(G)	GAA(E)
	CGC(R)							
Trimerization	AGA(R)	ATG(M)	AAA(K)	CAA(Q)	ATT(I)	GAA(E)	GAC(D)	AAG(K)
	ATT(I)	GAA(E)	GAA(E)	ATC(I)	CTT(L)	TCG(S)	AAA(K)	ATT(I)
	TAT(Y)	CAC(H)	ATC(I)	GAA(E)	AAT(N)	GAG(E)	ATT(I)	GCC(A)
	AGA(R)	ATT(I)	AAG(K)	AAA(K)	TTA(L)	ATT(I)	GGC(G)	GAA(E)
	CGC(R)							

measured to be around 687 resonance units (RU, where 1,000 RU corresponds to 0.1 degree shift in the SPR angle) through SPR analysis. In response to the addition of dimeric and trimeric EGFP:Cys proteins, increases in the RU value were observed up to around 1,160 RU and 2,521 RU, respectively. Theoretically, 1,000 RU is equal to the adsorption of around 1 nanogram of protein per square millimeter on the gold supports. Based on this, the density of the bound proteins such as monomeric EGFP:Cys, dimeric EGFP:Cys, and trimeric EGFP:Cys on the surface was estimated as around 6.87×10^{-10} g/mm^2, 11.6×10^{-10} g/mm^2, and 25.2×10^{-10} g/mm^2, respectively, indicating that the oligomerized proteins modified with cysteine residues could be densely packed due to the oligomerization states formed at gold surface. Orientation-controlled high density immobilization technique is the combination of cysteine- and oligomerization domain-based immobilization of protein. This method may be useful in gold-film biosensor applications, resulting in high density as well as well-oriented distribution simultaneously.

III. Conclusion

The selective immobilization of proteins to surfaces is one of the key processes for the successful development of protein detection systems, ranging from biosensor arrays to cellular targeting systems. Numerous studies have clearly indicated that properly controlled protein immobilization can improve the sensing activity on a solid support. Some factors such as correct and uniform orientation, minimum steric hindrance, and optimized buffer conditions must be taken into account to achieve ideal protein immobilization. To overcome these challenges in protein immobilization, future studies will focus on the development of better strategies effective for the controlled immobilization of conformationally ordered assemblies of proteins on a gold surface, potentially leading to an excellent performance of SPR measurement.

Acknowledgements

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