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A new method for multilayered, site-directed immobilization of antibody on polystyrene surface

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ABSTRACT

Polystyrene is a common substrate material for protein adsorption in biosensors and bioassays. Here, we present a new method for multilayered, site-directed immobilization of antibody on polystyrene surface through the linkage of a genetically engineered ligand and the assembly of staphylococcal protein A (SPA) with immunoglobulin G (IgG). In this method, antibodies were stacked on polystyrene surface layer by layer in a potential three-dimensional way and exposed the analyte-binding sites well. Enzyme-linked immunosorbent assay (ELISA) revealed that the new method showed a 32-fold higher detection sensitivity compared with the conventional one. Pull-down assay and Western blot analysis further confirmed that it is different from the ones of monolayer adsorption according to the comparison of adsorption capacity. The differentiated introduction of functional ligands, which is the key of this method, might offer a unique idea as a way to interfere with the dynamic behavior of a protein complex during the process of adsorption.

1. Introduction

The solid phase antibody used as probe is a key component in immunosensors and immunoassays microarrays, where the molecular recognition of target analyte by immobilized antibody to form a stable antigen–antibody complex and the separation of unbound immunoreagents from the immunocomplex are required [1–4]. In conventional method, antibodies were randomly bound to a certain surface (e.g., 96-well polystyrene microtiter plate employed in enzyme-linked immunosorbent assay) physically or chemically *via* surface modification (e.g., biotinylated, carboxylated and histidine-tagged) [4–6]. However, it has been found that such immobilized antibodies are active at a rate of only 5–10%, the majority of resident molecules on matrix surface is useless because its Fab fragment, which is indispensable in subsequent molecular recognition, may be denaturized or blocked during the attachment process, which makes them inaccessible to analytes [6,7]. In order to overcome these defects, oriented antibody immobilization was developed *via* an intermediate molecule (e.g., protein A or G), which binds to Fc fragment of antibody, making the rest of the molecule including Fab fragment exposed well, thus enhancing their accessibility to analytes [8,9]. This type of immobilization method can result in a significantly higher fraction of active antibodies, but usually suffers from a lower surface density of immobilized antibody since two immobilization steps are required [10,11]. Due to the limitation of two-dimensional (2D) space, the amount of antibody that can be immobilized on planer surface is usually low. To break through the bottleneck, porous substrates and three-dimensional (3D) polymeric networks have been proposed [9,12,13]. Undoubtly, these could significantly increase the immobilization capacity of antibody than that on 2D substrates. In addition to the sophisticated process of creating a 3D matrix, the major disadvantage is that it is more difficult to change buffers and recover trapped molecules because of the mass transfer limitation [11,14,15].

In this study, we established a new method of omni-directional antibody immobilization on polystyrene surface. First, a 12-mer affinity ligand for polystyrene surface from a phage display random library [16] was genetically recombined to SPA fusion protein. Next, by mimicking the molecular mechanisms of viable cells, two SPA and five IgG molecules can assemble a pod-shaped complex in an anti-parallel model (SPA containing five homologous IgG-binding domains: E, D, A, B and C, and each domain consisting of three α-helices, as illustrated in Fig. 1) [17–21]. The pod-shaped complex stands on polystyrene surface *via* the affinity ligand, thereby resulting in the formation of multilayered solid phase antibody in a site-directed manner. A double-antibody

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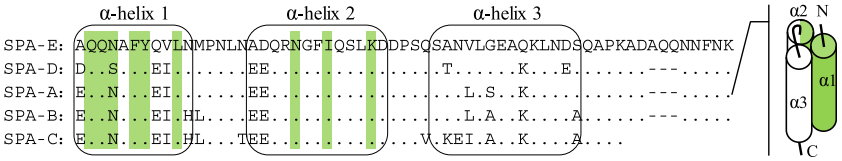

Fig. 1. Amino acid sequence of SPA's five IgG-binding domains (E, D, A, B and C) and the α-helix model of a single domain (on the right). The entire sequence of SPA-E is given and identical amino acids in other domains are depicted with a dot (.), while symbol (–) represents a vacancy. Positions of three α-helices in the domain structure are framed by boxes. Fc-interacting amino acid residues or regions are highlighted in cyan. It is because of the asymmetric distribution of Fc-interacting residues that produce the anti-parallel binding model with IgG.

sandwich enzyme-linked immunosorbent assay (ELISA) was developed for quantifying the active immobilized antibody using this novel method. Pull-down assay and Western blot analysis further confirmed that it might be quite different from planar immobilization according to the comparison of adsorption capacity.

2. Materials and methods

2.1. Preparation of ligand-linked SPA

The coding sequence of SPA (GenBank accession number: J01786.1, nt 292-1209) was cloned into the prokaryotic expression vector pET-21a allowing for bacterial expression of the SPA. An available affinity ligand for polystyrene surface screened by us from a phage display random library, named Lig1 (peptide sequence: FKFWLYEHVIRG) [16], was genetically fused to the N/C-terminus of SPA. To construct the plasmid encoding Lig1-SPA (Lig1 fused to the N-terminus of SPA), a *Bam*H I-*Bam*H I fragment was prepared by annealing the sense (5′-GATCC TTC AAA TTC TGG CTA TAC GAA CAT GTA ATA CGG GGG G-3′; the underlined sequence corresponding to Lig1) and the antisense (5′-GATCC CCC CCG TAT TAC ATG TTC GTA TAG CCA GAA TTT GAA G-3′). The annealing procedure was performed by incubating the pair of single-strand DNAs at 95 °C for 15 min followed by its cooling down to 30 °C within 60 min. The *Bam*H I-*Bam*H I fragment was then inserted at the *Bam*H I site of pET-SPA (the resulting plasmid named pET-Lig1-SPA). To construct plasmid encoding SPA-Lig1 (Lig1 fused to the C-terminus of SPA), a *Xho* I-*Xho* I fragment obtained by annealing 5′-TCGAG TTC AAA TTC TGG CTA TAC GAA CAT GTA ATA CGG GGG C-3′ and 5′-TCGAG CCC CCG TAT TAC ATG TTC GTA TAG CCA GAA TTT GAA C-3′ in the same way, was introduced into pET-SPA predigested with *Xho* I (the resulting plasmid named pET-SPA-Lig1). After the recombinant expression plasmids were transformed into *Escherichia coli* BL2l competent cells, the N- and C-terminal Lig1-tagged fusion proteins were induced at 37 °C by using 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). Next, cells were harvested by centrifugation for 15 min at 5000×*g*, and the pellet was suspended in an equal volume of ice-cold phosphate-buffered saline. After sonication on ice (4 times for 30 s each at 48–56 W power and 30 s pulse in between), the cell homogenate was centrifuged at 15,000×*g* for 15 min. Proteins from supernatant fraction were purified by using Talon metal affinity resin (BD Biosciences) and determined by performing a Western blot.

2.2. Antibody immobilization and Hepatitis B surface antigen (HBsAg) ELISA

Two types of specific anti-HBsAg antibodies are employed in HBsAg ELISA: monoclonal antibody for immobilization, and the polyclonal one conjugated to horseradish peroxidase (HRP). Anti-HBsAg monoclonal antibody, and N- and C-terminal Lig1-tagged SPA, at a molar ratio of 5:1:1, were added to a siliconized eppendorf tube with a binding buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1% BSA, 1 mM dithiothreitol, 0.05% Nonidet P-40, plus antiproteases) and incubated for 1 h at 4 °C on a turntable. After resting for another 1 h, this binding solution was added to polystyrene 96-well microplate (Nunc, Denmark) with 100 μL per well for protein coating. Plates were incubated for 8 h at 4 °C, the solution was removed and replaced with a washing buffer (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). After rinsing extensively, the plate was ready for detection of target analytes according to the routine protocols.

Load 100 μL per well of serially diluted HBsAg into the coated plate, followed by incubation at 37 °C for 0.5 h. After washes with 300 μL per well of washing buffer, 100 μL of HRP-conjugated anti-HBsAg polyclonal antibody at 1:3600 dilution, was transferred into each well and incubated for another 0.5 h. During the assay, monoclonal and polyclonal antibodies reacted with the existing HBsAg to form an "antibody-HBsAg-antibody-HRP" immune complex. After the unbound conjugates were washed off, tetramethyl benzidine (TMB) was applied to indicate the test result by measuring the absorbance value at 450 nm (A_{450}) using a model 550 microplate reader (Bio-Rad, USA). All tests were repeated four times and the arithmetic mean of A_{450} was calculated.

2.3. Pull-down assay with polystyrene beads

Polystyrene beads (another form of polystyrene surface, 1 μm in diameter; Sigma–Aldrich, USA) were transferred into the binding solution (containing N- and C-terminal Lig1-tagged SPAs, and Monoclonal antibody at a molar ratio of 1:1:5), mixed, and incubated with tumbling for 2 h at 4 °C. After being washed with washing buffer, proteins bound to polystyrene beads were released by boiling in SDS sample buffer and analyzed by a Western blot. For Western blot, proteins were separated in a 10% non-reducing SDS–polyacrylamide gel and then transferred by electroblotting to a polyvinylidene difluoride (PVDF) membrane. The membrane was pre-blocked with dried milk in TBS for 20 min, hybridized with HRP-conjugated anti-mouse IgG antibody (1:4000 diluted in the milk block) for 2 h at room temperature, washed in TBST (TBS containing 0.1% v/v Tween 20) and developed with 3,3′-diaminobenzidine (DAB). Densitometric analysis of the hybridizing bands was done with the software Gel-Pro Analyzer (Media Cybernetics).

3. Results and discussion

For the expression of N/C-terminal Lig1-tagged SPA, both pET-Lig1-SPA (encoding Lig1-SPA) and pET-SPA-Lig1 (encoding SPA-Lig1) were constructed from the original plasmid pET-SPA, as illustrated in Fig. 2. Subsequently, the resultant plasmids and the original one (as a control) were transformed into *E. coli* BL21 for protein expression. After purification, expression products were confirmed by Western blot using a monoclonal anti-SPA antibody that can specifically recognize SPA (Fig. 2). We may notice that, compared to the original SPA, the molecular weights of Lig1-SPA and SPA-Lig1 obviously increased with the insertion of Lig1.

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