Food Engineering Progress
Vol. 7, No. 4. pp. 189~194 (2003.11)

총 설

Immobilization and Detection Techniques for Biosensors

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Abstract

The biosensor technology, which makes it possible to detect biomaterial such as protein, pathogen, and small molecules, is useful in such areas as diagnosis, bioprocessing, and food analysis or safety. For the development of a highly sensitive biosensor, immobilization techniques of organic/bio films on solid substrate, and detection methods of protein-protein reactions appearing in a few nanometers region from the sensor surface should be established. In this review, several immobilization techniques and detection methods are reviewed based on the articles reported recently.

Key words: biosensor, detection method, immobilization of protein

Introduction

Biosensor has made it possible to analyze several protein-protein, protein-molecules, and protein-pathogen bindings on solid surface. For the successful development of biosensor, three key elements, a design of protein probe, an immobilization of the protein probes, and a detection of protein-protein or protein-small molecules binding should be achieved. Of them, the protein probe, of which a monoclonal antibody is representative, is the receptor that can specifically bind with a target material.

The strategy for immobilization of proteins on a solid substrate is divided into two categories, that is, a physical binding and a chemical binding, Although the physical binding provides simple processes, it has several disadvantages; denature of proteins and poor reproducibility. As an alternative to the physical binding, the chemical binding, which resulted in a covalent bonding between protein and a sensor surface, has been prevalently adopted recently. In addition of a simple immobilization of protein, it is necessary to fabricate organic/bio films that can preserve their folded conformation of proteins, and control the orientation of them on a solid substrate, in order to improve reactions between receptors and analytes. However, it is not possible to fabricate organic/bio films by physical/chemical techniques including the conventional vacuum evaporation, sputtering, chemical vapor deposition (CVD), and molecular and beam epitaxy, which has been successful in optoelectronics and semicoductors. Instead, for the well-ordered and stable organic/bio films, nanofilm-fabrication techniques such as a self-assembly technique and a Langmuir-Blodgett (LB) technique can be applied. Using these techniques, high-quality and ordered nano-scale organic/biofilms can be fabricated without the loss of specific functionality.

On the other hand, since binding reactions between receptor and analyte which appears a few nanometers region from the surface, detection methods sensitive enough to changes of properties such as mass and refractive index in nanometer-scale regions should be developed. At present, a fluorescence microscopy bound with the sandwich methods derived from an immunoassay is diversely applied for the detection of binding of protein to a sensor surface. Typically, a substrate is either directly probed with a fluorescent molecule or in two steps by first using a tagged probe, which can then be detected in a second step using a labeled affinity reagent.

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Thus, label-free methods based on electric, optical, and mechanical principles have advantages as a direct approach for detection of receptor-analyte binding.

In this article, several fabrication techniques of protein layers and their applications and non-labeling detection method are centered on the article published recently.

Nanofabrication of Biofilm

Self-assembly

Molecular self-assembly is currently the most advanced development for fabrication of nanometer-scale biomolecule layers, and is the spontaneous association of molecules under equilibrium conditions into stable, structurally well-defined aggregates (Timp, 1998). Many self-assembly systems have been investigated, but monolayers of alkanethiolates are probably the most studied self-assembly monolayers (SAMs) to date. The SAMs of alkanethiols and disulfides form organic interfaces on gold, with their properties controlled largely by the end groups of the molecules comprising the film (Bain, et al, 1989; Prime and Whitesides, 1991). The success of organo-sulfide SAMs on noble metal surfaces is due to several reasons; the simplicity and reliability of the method of preparation; reproducibility of the generated surfaces; the flexibility of generating a wide variety of surfaces via the incorporation of different groups into the alkyl chain and chain termini of a SAM; and the possibility of applying a wide variety of techniques to the characterization of the SAM surface (Ferreti, et al., 2000).

Two accesses can be considered; 1) use of carboxylate-terminated alkanethiols layer (Fig. 1 a), 2) use of a thiolated protein (Fig. 1 b), in order to fabricate the protein, that is, receptor layer on a gold surface via the self-assembly technique. In the former method, firstly a SAM of carboxylate-terminated alkanethiol is self-assembled. Following esterfication of the carboxylic groups, a protein layer is fabricated via the chemical bonding between the ester functionality and lysine residues of the protein. The enzyme catalase layer was formed on a modified substrate of homologous and mixed SAMs of the two carboxylate-terminated alkanethiols, 3-mercapto-

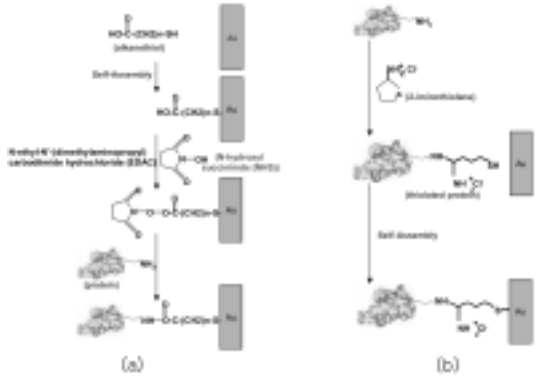

Fig. 1. Fabrication of protein layer using the self-assembly technique. (a) After alkanethiol self-assembled on a gold surface, the protein layer is immobilized on it. (b) After the thiol group attached to protein molecule, it is immobilized via using the self-assembly technique.

propanoic acid (3-MPA), and 11-mercaptoundecanoic acid (11-MUA) (Patel, *et al.*, 1997).

In the latter method, after the protein is modified into one with thiolated group via the chemical reaction between lysine residues of the protein and some chemicals such as a 2-iminothiolane or a N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), the thiolated protein is self-assembled on a gold surface (Park and Kim, 1998).

In our laboratory, in order to develop the biosensor for detection of Escherichia coli O157:H7 pathogen, the antibody was fabricated on gold surface according to the former method (Oh, *et al*., 2002). Especially, the binding protein layer (protein G) was inserted in between the SAM and the antibody layer for the well-orientation configuration, as shown in Fig. 2 (a). Also, the SAM was fabricated using a mixing solution of a hexanethiol and a MUA for minimizing a steric hindrance by antigen size. As shown in Fig. 2 (b), an antibody-antigen reaction varied according to a ratio of 11-MUA to hexanethiol. That meant that antigen's accessibility to the antibody's fab domains can be improved by spacing among antibodies.

As the strong technique for functionalisation of solid surface, the self-assembly technique will be more and more attractive in molecular electronic devices, and in the assembly of supra-molecular structures as well as in sensors.

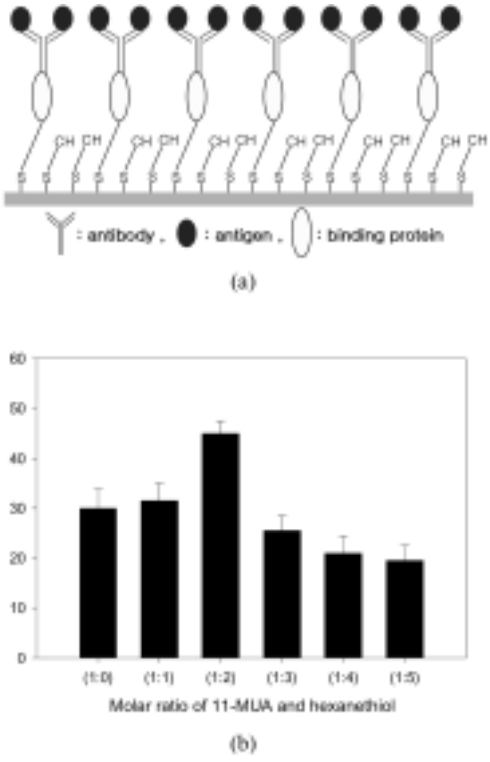

Fig. 2. Immobilization of antibodies on the self-assembled monolayer (Oh, *et al.* (2002)). (a) The protein layers immobilized using a mixed SAM for minimizing hindrance. (b) The changes of the SPR minimum position shift by reaction between antigen and antibody immobilized onto the protein G layer chemically bound to the self-assembled monolayer with various molar ratios of 11-mercaptoundecanoic acid (11-MUA) and hexanethiol.

Langmuir-Blodgett technique

Langmuir-Blodgett (LB) films are formed on the surface of solid substrates by consecutive crossing or consecutive contact of a water surface on which a monomolecular layer, called as a Langmuir layer, has been formed previously. The deposition method that consists of consecutive crossings by a plate of water surface with the Langmuir layer formed was devised by Blodgett and is referred to as the LB method or the method of vertical lift. Application of the LB technique for fabrication of nano-films provides several advantages; 1) precise ordering of molecule layers, and 2) controlling the thickness of thefilms.

After it was showen that the orientation of an antibody could be controlled by surface pressure, several antibody layers were fabricated via the LB technique (Turko, *et al*., 1992; Tronin, et al., 1996; Preininger, *et al*., 2000). Also, as the other method for the orientation of an antibody, the antibody was immobilized on the substrate on which the binding proteins such as protein A and protein G were fabricated with the LB technique (Owaku and Goto, 1995; Dubrovsky, *et al*., 1996).

Detection method

Surface plasmon resonance

The surface plasmon resonance (SPR) phenomenon can be very useful in the field of affinity biosensing since the plasmon dispersion relation (PDR) is very sensitive to the localized index of refraction at the metal's surface (O'Brein, *et al*., 2001). SPR biosensors have become an established method of measuring molecular interactions. Biosensor experiments involve immobilizing one reactant on a surface and monitoring its interaction with a second component in solution. SPR biosensors measure the change in refractive index of the solvent near the surface that occurs during complex formation or dissociation. The instruments are capable of characterizing binding reactions in real-time without labeling requirements. Consequently, the SPR biosensors can be used to study the interactions of any biological system from proteins, oligonucleotides, and lipids to small molecules, phage, viral particles, and cells (Green, *et al*., 2000). Also, kinetics of protein deposition on a solid substrate can be in situ monitored using SPR.

The typical SPR system based on the Kretschmann prism is shown in Fig. 3 (a). As the incidence angle of the laser is scanned in some ranges, the intensity of the reflected light from chip is measured by photodetector such as photomultiplier tube (PMT). Properties change in a few nanometers near a substrate, that is, protein-protein reaction can be identified via the changes of the incidence angle called as SPR angle, in which the intensity is minimized. More reviews of principle and application of the SPR system were reported, elsewhere (Salamon, *et al*., 1997; Homola, *et al*., 1999; Mullet, and Yeung, 2000).

In our laboratory, the SPR was applied to a fabrication

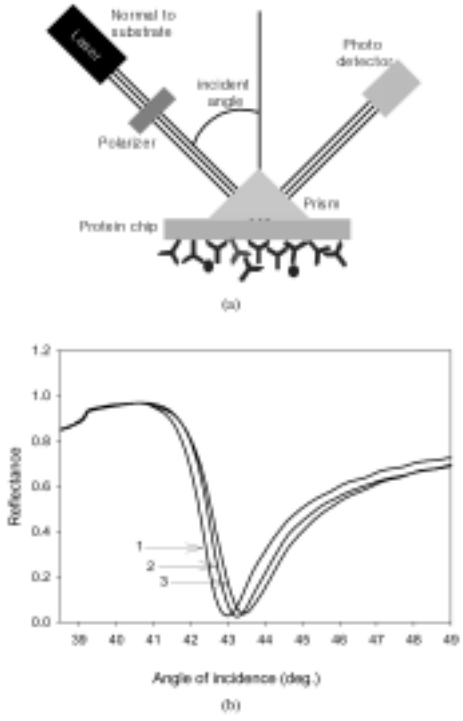

Fig. 3. Surface plasmon resonance (SPR) system for detection of protein-protein or protein-small molecules binding (Oh, et al., 2002). (a) Configuration of the SPR system using the Kretschmann prism. (b) The SPR curves corresponding to the deposition of organic/bio layers on gold surface, 1. 11-mercaptoundecanoic acid, 2. protein G, 3. antibody.

of immunosensor, and a detection of protein-protein reaction on the sensor surface. The changes of SPR angle according to the deposition of thin films on the gold surface were shown in Fig. 3 (b). Considered that 11-MUA is about 1.5 nanometers thick, the SPR analysis could be high sensitive to organic thin films.

Recently, a SPR imaging (SPRI), which provides lateral resolution about objects, was developed. The SPRI was successfully applied to imaging of the patterned monolayer of biopolymers successfully, and will be useful as the detection method for biosensor since its inherent advantage (Jordan and Corn, 1997; Steiner, *et al*., 1999).

Ellipsometry

Because an ellipsometry, which uses the physical phenomenon that a thin film modifies the polarization state of an elliptically polarized light, is the technique for acquiring optical properties and structure of single or multi-layer thin film layers on substrates, it is high sensitivity enough to properties changes of thin layers on substrates, it was applied to detection of protein-protein reaction. The several configuration of an ellipsometry have been developed for measuring changes of phase angle and intensity of reflectivity of some films on a substrate (Arwin, 2000). The prevalent configuration is null-ellipsometry type shown in Fig. 4(a). The ellipsometry have been mainly applied to adsorption of proteins on substrates (Arwin, 1998; Elwing, 1998; Benesch, *et al*., 35; Turko, *et al*., 1992). The ellipsometry as a detection method for a biosensor have been never applied to detection for antigen-antibody reaction on a solid substrate (Mandenius and Mosbach, 1988). Recently, imaging ellipsometry (IE), in which a photo-detector was replaced by a CCD camera, was applied to the detection of protein-protein binding on a solid surface (Jin and Tengvall, 1995).

In our laboratory, the detection of bindings of biomaterials such as microorganism and protein are being investigated using the imaging ellipsometry system based on the off-null ellipsometry. Fig. 4 (b) is an ellipsometric image of protein G spots formed on a gold surface on which 11-MUA was deposited with the self-assembly technique. The IE, together with the SPRI will be recognized as the novel applicable tools for the simultaneous detection of several protein-protein binding.

Scanning probe microscopy

The common principle of the different adaptation of scanning probe microscopy (SPM) is the scanning of the sample by a very fine probe (cantilever tip with a radius of curvature below 100nm and a cantilever length of 100 and 200 um, respectively) at a very small distance. A vacuum is not required and biological objects can be imaged in their physiological environment. Various physicochemical probes have been adapted for the SPM. Atomic forced microscopy (AFM) is the most widely used technique for analyzing the immobilization of biomolecules at the surface (Pereira, 2001). Using this technique, the topography of the surface is probed by

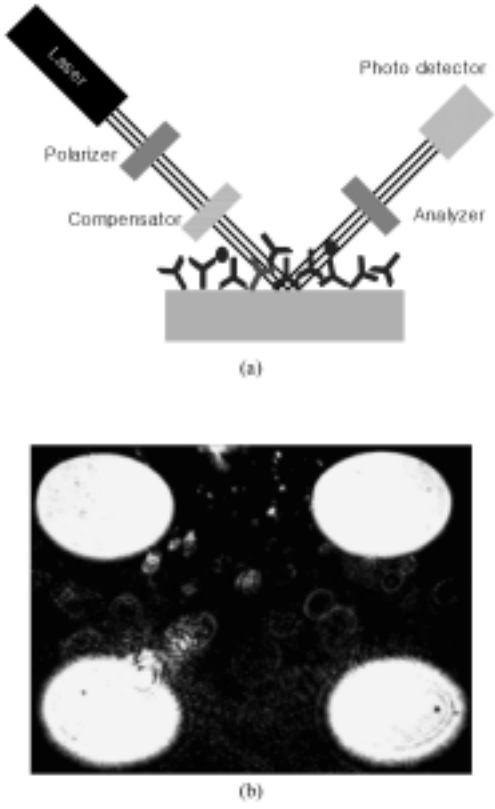

Fig. 4. Ellipsometry for detection of protein-protein or protein-small molecules binding (Bae, *et al.*, 2003). (a) Configuration of null type ellipsometry. For imaging ellipsometry, the photodetector was replaced by CCD camera. (b) Ellipsometric image of protein spots on gold. The spot size is about 120 μm.

interaction forces between the fine tip and the molecules at the surface. With optimized scanning modes and tips, the surface of soft materials such as hydrated polymers or proteins can be imaged with a lateral resolution in the nanometer scale, i.e. with molecular resolution. Thus, this technique is extremely valuable for analyzing the formation, the molecular arrangements, and the destruction of surface architecture.

In recent, a micro-mechanical cantilever sensor using a shift of mechanical resonance of the cantilever of the AFM by mass adsorption appeared as a novel detection mechanism for a nano-scale biosensor or biochip (Raiteri, *et al.*, 2001). Since the cantilevers of the sensor system are prepared using the silicon micro-machining technique, the minimization and array-configuration are possible, and a shift of their resonance frequency is measured via the detection method of the cantilever bending used in the AFM. Receptor-analyte bindings were detected using the micromechanical cantilever sensor, and the array system was realized (Wu, *et al.*, 2001; Mckendry, *et al.*, 2002).

Future perspectives

Biosensor technology provides a powerful and versatile tool for detection of chemical or biological materials in the medical, biological, and food industry areas. For successful development of biosensor, not only a design of high specific protein probes, but also immobilization protocols of the protein probes on solid substrate, and its detection methods should be established in parallel.

For a successful immobilization of protein, fabrication processes of nanometer-scale organic or protein films such as the self-assembly or the Langmuir-Blodgett technique are required. Also, further studies for the control of the orientation of proteins in a few nanometer ranges near surface should be executed. In addition, an integration technology of protein spots in a solid substrate, including spotting of the proteins in nanometer scales, might be developed.

At present, the surface plasmon resonance (SPR) technique is being successfully applied to the detection of protein-protein or protein-small molecules in nanometer-region from a substrate. However, the conventional SPR system is inappropriate for multiple sensing on a sensor surface. Thus, imaging-based methods such as SPR imaging and imaging ellipsometry were proposed. Though they are promising detection methods for multiple sensing, the sensitivity level should first be improved for practical application. Also, the hardware and protocols of detection systems should be simplified for simple operation.

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