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Surface modification methods to improve behavior of biosensor based on imaging ellipsometry

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ABSTRACT

Surface modifications not only perform functions as ligand immobilization and biosensing interface formation, but also play a crucial role for inhibiting noise and enhance biosensor signal. After years of development, several surface modification methods designed for the biosensor based on imaging ellipsometry have become more and more practical and already been successfully implemented in the detection of various biomolecules. Some typical surface modification approaches are reviewed.

1. Introduction

Ellipsometry is a nondestructive optical technique and conventionally used to determine film thickness beyond a resolution of 0.1 nm [1]. Imaging ellipsometry is an enhancement of standard single-beam ellipsometry that combines the power of ellipsometry with microscopy. Compared to the conventional ellipsometry, imaging ellipsometry inherits the advantage of the spatially high vertical resolution and also owns a large field of view [2]. These features give imaging ellipsometry the capability to visualize the thickness distribution of a large area quantitatively, and provide the potential to form a label-free and highly sensitive platform for multi-protein parallel analysis.

The biosensor concept based on imaging ellipsometry (BIE) for the visualization of biomolecule interactions has been reported since 1995 [3]. Imaging ellipsometry is used to observe the distribution of protein layer thickness on a patterned surface and its principle to detect protein interactions is shown in Fig. 1 [4]. A ligand is immobilized on a surface for biosensing; while a receptor exists in an analyte solution. When the biosensing surface composed of ligands is exposed to the analyte solution, ligands and receptors can interact with each other to form bio-complexes due to their affinity. The thickness of protein layers on a surface will change during the reaction process. With the visualization of imaging ellipsometry, the change is able to determine quantitatively [3], and in this way, the existence of the receptor in the analyte solution can be verified.

The first experimental model of imaging ellipsometry reader in BIE system is a conventional polarizer-compensator-sample-analyzer null ellipsometer and its schematic diagram is shown in Fig. 2 [2,3]. When it is used to visualize layer thickness distribution of a large square, it is operated under the null and off-null mode, which almost fulfills the null condition on bare substrate and detects layer sample area with off-null ellipsometric principle. In order to optimize the ellipsometric contrast between the adsorbed layers and the substrate, detection is performed close to the angle of incidence of the pseudo-Brewster angle of the bare substrate [2]. Considering the dispersive effect to improve imaging spatial resolution, in the recent decade, it has been developed as a spectroscopic imaging ellipsometry system which has the lateral resolution of optical imaging ellipsometry with various wavelengths [5].

Accompanying with the update of imaging ellipsometry setup, an integrated microfluidic system [6,7] with 48 independent channels has been developed to function as solution delivery and bioreactor in BIE. In this microfluidic system, it is much easier and faster to fabricate protein microarray with the advantages of inerratic pattern, homogeneous spotting and low consumption. With the optimization of imaging ellipsometry setup and practice on biomolecule reactor, BIE has become a practical automatic protein microarray technique and accumulated some successful application experiences in the field of biology and biomedicine, for instance, the clinical diagnosis for hepatitis B [8], cancer marker tests [9–12], bacterium and virus detection [13–15], and screening and identification of antibodies [16].

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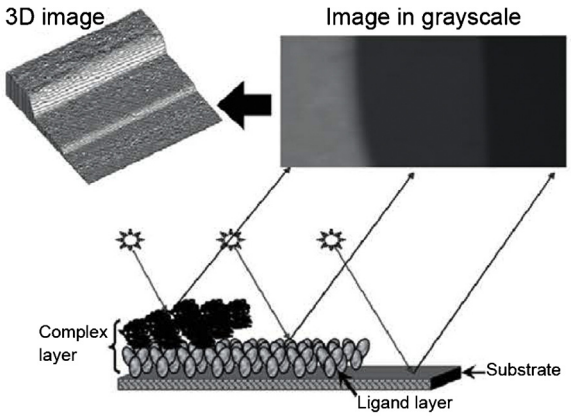

Fig. 1. The principle of BIE to detect protein interactions. The incident wave of polarized light irradiates the protein layer and the reflective beam carries its information, which can deduce the thickness of the protein layer. After ligands react with receptors, the biosensing interface includes three parts: the bear substrate, the ligand layer and complex layer, which can be seen in ellipsometric image with the value of reflection intensity in grayscale. The variation of the protein layer thickness causes the value in grayscale of the image changed. The thickness distribution of protein layers can be easily seen in three dimensions.

For detecting protein interactions with BIE, the general procedure requires the immobilization of ligand on the biosensing interface to form the sensing surface and then the addition of analyte solution to identify the existence of receptors by the specific recognition against ligands [4,17]. Since BIE sensitively responds to even tiny change of layer thickness in the order of 0.1 nm, not only the signal contributed by specific binding but also any undesirable adsorption on the biosensing surface affects BIE result obviously. In order to improve BIE performance, it brings forward a high request to the property of biosensing surface. On the one hand, the biosensing surface should present the bioactivity of ligands well to capture receptors with good reproducibility and stability. On the other hand, it must suppress the negative influence caused by non-specific adsorption and maintain the resolution of imaging ellipsometry.

Polished silicon wafers have been and are still widely used as the substrates in ellipsometric measurements due to the features of high reflective index, appropriate polarity and fine planeness. However, natural silicon surface neither possesses the ability to immobilize a protein ligand to present its good bioactivity or preserves non-specific adsorption to reduce negative impacts. For this reason, silicon wafers could not be available as BIE substrates directly. Since surface chemistry modification can be easily carried out on silicon surface by silanization reaction [1], it is a productive shortcut to rely on surface modification approaches to change the property of silicon wafers, rendering them unique characteristics

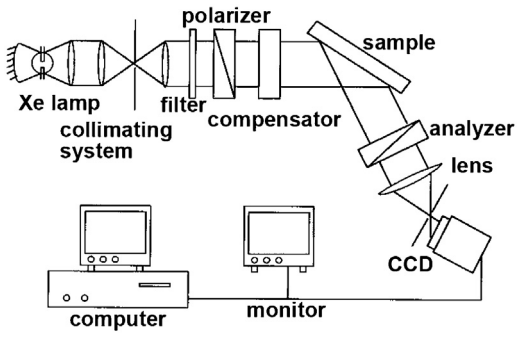

Fig. 2. Schematic diagram of an imaging ellipsometer with an expanded beam, fixed polarizing components, and a CCD camera.

to fit the request of BIE biosensing surface. In this article, several surface modification methods for silicon wafers which have been used in BIE applications are reviewed.

2. Silicon surface modification methods for BIE

2.1. Physical adsorption

In its initial stage, the ligand immobilization is performed by physical adsorption. Silicon wafers cleaned by piranha solution are incubated in the solution of dichlorodimethylsilane (DDS) for a few minutes to form a highly hydrophobic surface [3,18] with the advancing contact angle of 80°. Protein ligands can be immobilized on a silicon surface mainly through hydrophobic interaction, and construct the sensing surface. Fig. 3 is an example to adsorb different proteins on DDS modified surface [3]. After the immobilization of immunoglobulin G (IgG), human serum albumin (HSA) and fibrinogen (Fib) on the modification surface, the silicon substrate is incubated into an anti-IgG solution. A thickness increase only occurs in the areas formed by IgG, while almost no change can be observed on HSA and Fib surface. It proves that IgG immobilized on DDS modified surface can present its bioactivity and bind anti-IgG with good specificity.

Physical adsorption is a convenient approach to bind protein ligands, and normally the adsorbed amount on hydrophobic surfaces is not less than that on hydrophilic surfaces. However, ligand assembled by this method may result in a partial denaturation and can easily be removed from the sensing surface even by slight but indispensable rinsing [19,20]. In addition, the adsorbed protein ligand can be displaced from the surface by more active proteins due to competitive adsorption [21,22], which would influence the bioactivity presentation and result in instability in practice.

2.2. Covalent immobilization

To overcome those problems in the ligand immobilization with physical adsorption, the concept of covalent immobilization is introduced to immobilize ligands to construct biosensing surfaces. Referring to several methods which are successfully used in other immunoassay techniques [23,24], we have investigated the feasibility of using 3-aminopropyltriethoxysilane (APTES) to modify the silicon surface and then reacted with glutaraldehyde (Glu) or succinic anhydride to immobilize protein covalently for BIE [25].

2.2.1. Modification with APTES and Glu

Silicon wafers with native oxides are washed in piranha solution to remove contaminants and improve the amount of silanol groups. Then, silicon wafers are incubated in APTES solution for 2 h to produce —O—Si(OC_2H_5)$_2$—(CH_2)$_3$—NH_2, forming a layer of densely packed amino groups on the silicon dioxide layer [25]. Then, the silicon wafers are treated with Glu solution for 1 h, and during this period, Glu reacts with amine groups on a surface modified with APTES to fabricate —(CH_2)$_3$N=CH(CH_2)$_3$—CHO for capturing amino groups of protein ligands [25].

2.2.2. Modification with APTES and succinic anhydride

A surface modification procedure with APTES and succinic anhydride is similar to that using APTES and Glu to modify silicon surface [15,16]. The difference is that the silicon wafers are incubated in a saturated solution of succinic anhydride instead of glutaraldehyde, after the treatment of APTES. Succinic anhydride reacts with amine groups of–O—Si(OC_2H_5)$_2$—(CH_2)$_3$—NH_2 on the surfaces and forms —(CH_2)$_3$NH—CO(CH_2)$_2$—COOH. Before the application to immobilize protein ligands, the silicon surface needs to be activated by 0.05 mol/ml N-Hydroxysuccinimide (NHS) and 0.2 mol/ml 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC)

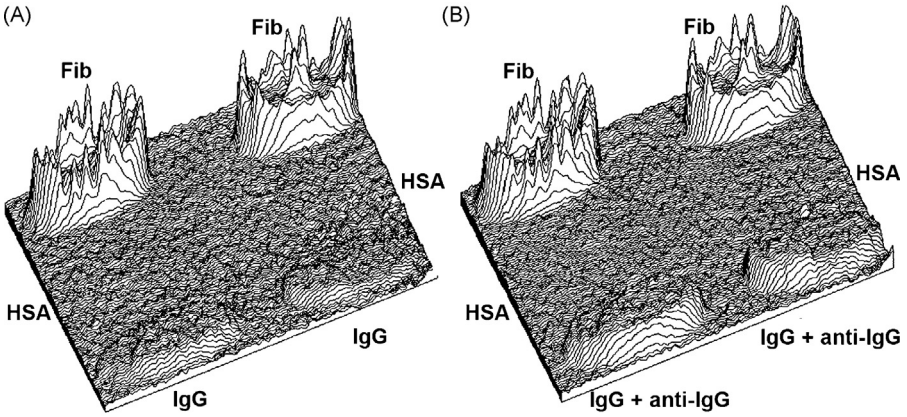

Fig. 3. Proteins adsorption on silicon surfaces modified with DDS. (A) The dots of absorbed layers of Fib, HSA and IgG on the modification surface. (B) After incubation in anti-IgG serum.

to transfer carboxyl group to sulfo-NHS ester, which could covalently bind amino group of proteins efficiently.

The modification surfaces become much more hydrophobic by the covalent immobilization method, which the advancing contact angle decreases to 50° on APTES-Glu surface. Compared to a surface modified with DDS, these two modification strategies lead to better stability and improved bioactivity for protein ligands immobilized on silicon surfaces. Fig. 4 [25] reveals that the advantages of APTES-Glu modification surface by comparing IgG immobilization amount and anti-IgG capture capability on DDS and APTES-Glu modification surfaces. However, the strong covalent binding and the high surface concentration of ligands may make ligands denatured and also lead to higher steric hindrance.

2.3. Ligand surface concentration control with a mixed silane layer

The ligand surface concentration is a crucial factor for the BIE performance, since it can have a direct influence on the amount of analyte capture. In order to optimize the ligand surface concentration, methyltriethoxysilane (MTES) is mixed with APTES to modify the silicon surface under different conditions [26], forming a surface composed of a mixture of amino and methyl terminations. Methyl cannot interact with Glu and only amino groups react with Glu to create binding sites for ligands.

Silicon surfaces are exposed to a fresh ethanol solution of APTES and MTES (1:30, mol/mol). This procedure renders the surface medium hydrophobic. Then the surface modified with the mixed silane is incubated in Glu solution. Since Glu reacts with amino groups, proteins could be only covalently immobilized on the surfaces occupied by APTES rather than MTES. In this case, the saturated ligand surface concentration decreases remarkably, leaving sufficient spaces for ligand molecules to keep their structure. Compared with the surface modified with APTES, protein ligands covalently immobilized on mixed silane surfaces should retain their bioactivity well and recognize more target molecules.

The performance of surfaces modified with APTES/MTES-Glu and APTES-Glu has been compared in Fig. 5 [26]. The amount of IgG covalently immobilized on these two modified surfaces is controlled to be the same level. The capability to capture anti-IgG on APTES/MTES-Glu modified surfaces is remarkably overwhelming than that on APTES–Glu modification surfaces, suggesting IgG presents better bioactivity on the APTES/MTES-Glu modified surface.

2.4. Prevention of non-specific binding

Non-specific adsorption is a tremendous barrier to prompt BIE application in the fields of biomedicine and clinical. Non-specific adsorption is mainly caused by physical adsorption of protein

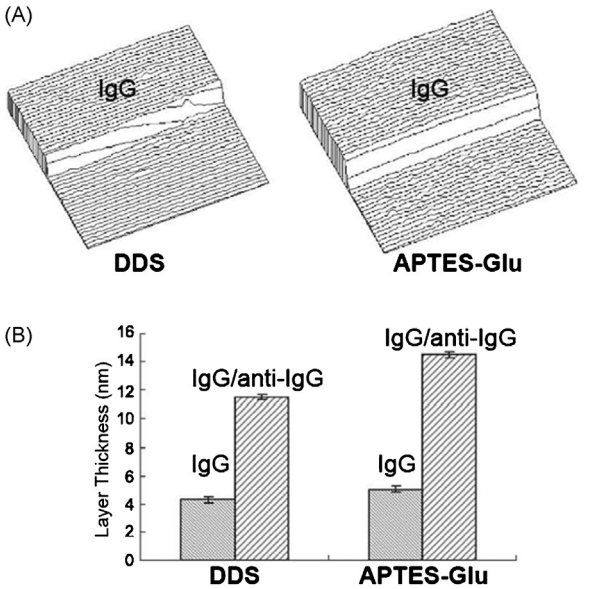

Fig. 4. Comparison between DDS and APTES-Glu modification surfaces. (A) The saturated monolayer of IgG on the silicon surface modified with DDS and APTES-Glu. (B) Layer thickness of IgG and IgG/anti-IgG complex on DDS and APTES-Glu surfaces. The amount of both IgG saturated monolayer and anti-IgG bound with IgG immobilized on APTES-Glu surface is higher than that on DDS surface.

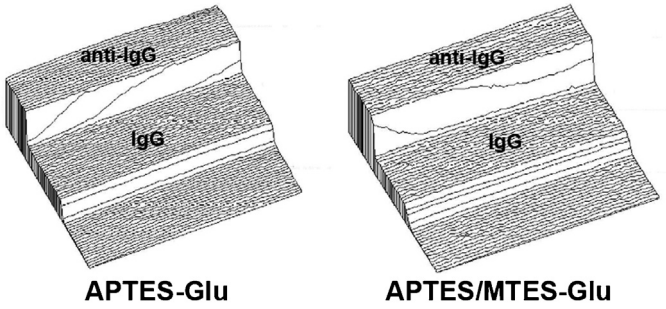

Fig. 5. IgG on APTES-Glu and APTES/MTES-Glu surfaces bound with its antibody. The amount of IgG covalently immobilized on these two surfaces is controlled to be the same level and the IgG layer thickness is about 5.0 ± 0.5 nm. The amount of bound anti-IgG is about 11 ± 0.7 nm and 9.5 ± 0.5 nm on APTES/MTES-Glu and APTES-Glu surfaces, respectively.

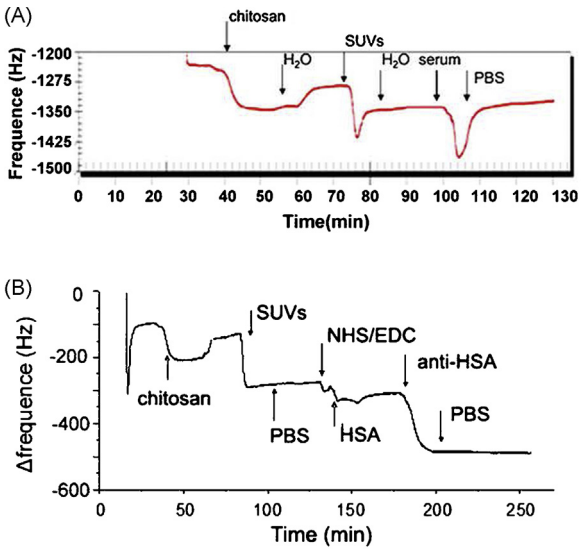

Fig. 6. QCM real time results to evaluate the performance of phospholipid membrane formed on chitosan modified surface. (A) Phospholipid membrane formation on chitosan and its reaction with serum samples. (B) HSA binding on phospholipid membrane.

molecules on solid surfaces, and it may bring an obvious signal even under the situation that the target molecule does not exist in the analyte solution. Poly ethylene glycol (PEG) and lipid bilayers have been used to modify surfaces to prevent non-specific adsorption, and present good results in previous reports [27–29]. The two compounds are tested to constitute a modified silicon surface to reduce the effect of non-specific adsorption.

A supported phospholipid bilayer is fabricated on an APTES-Glu modification silicon surface. The phospholipids bilayer is prepared by covalent immobilization of an L-α-phosphatidylethanolamine layer on the silicon surfaces and followed by incubation with PEG functionalized phospholipids in aqueous solution. The terminal active group on long arm chain of PEG is used for immobilizing ligand proteins covalently. Since lipids can block the silicon surfaces and suppress non-specific binding, a mixed phospholipids bilayer composed of methyl termination and carboxyl termination PEG functionalized phospholipids is fabricated [30].

Despite of good performance on suppressing non-specific adsorption, lipid bilayer modification methods are impeded in the further application by the poor air stability. In order to break this limitation, chitosan is introduced to fabricate an air stable phospholipid membrane on silicon surfaces [31]. Chitosan is immobilized on the APTES-Glu modified surface to provide a suitable condition for lipid membranes to enhance their air stability. Phospholipid membrane is formed by small unilamellar vesicles (SUVs) composed of 1,2-dimyristoyl-sn-glycero-3-phophatidylcholine (DMPC) and 1,2-dilauroyl-sn-glycero-3-phosphatidylethanolamine (DLPC) adsorption and then rupture on the chitosan surface. For covering membranes with polymers, chitosan is linked with DLPC through Glu as crosslinker. After chemical activation, ligands can be immobilized on this membrane surface.

Fig. 6 [31] shows QCM results of the reaction against serum samples and the interaction between HSA and its antibody on the phospholipid membrane. When serum passes through the modification surface, the frequency level drops greatly due to the adsorption of biomacromolecules from serum. However, the frequency can recover to the original level of lipid membrane after rinsing with PBS, indicating the adsorbed molecules can easily be washed off. This proves that the serum is resisted by polymer shielding lipid membranes. For the immobilization of HSA on the modified surfaces to recognize its antibody, there is no obvious fluctuation in frequency during a long time rinsing procedure, presenting functionalization of ligand immobilization on modified surfaces as well as good stability of the protein and phospholipid complex

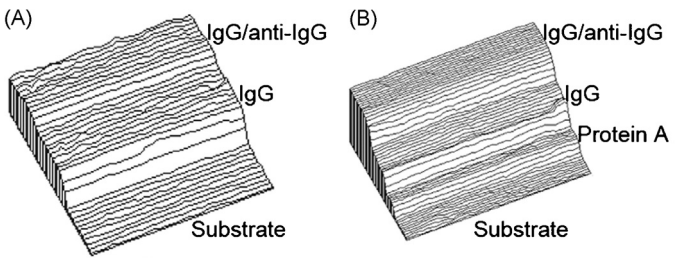

Fig. 7. Comparison of the amount of bound anti-IgG on IgG sensing surface constructed by physical adsorption and oriented immobilization of protein A. (A) IgG bound with anti-IgG immobilized by physical adsorption on silicon surface. (B) IgG bound with anti-IgG oriented immobilized by protein A on the silicon surface. The amount of IgG bound on the surface modified with protein A is much more than that on DDS-modified surface.

2.5. Oriented immobilization

In order to have some better bioactivity presentation of ligands, especially for antibody ligands which are one of the most popular ligands used in clinical detections, an oriented immobilization of ligands is requested. Protein A could bind selectively the Fc domain of antibody molecules [32] and expose the Fab portion outside where specific recognition occurs. This feature is applied to design a method for the oriented immobilization of antibody ligands.

Fig. 7 [18] demonstrates the feasibility of protein A for the oriented immobilization of IgG on the silicon surface. A hydrophobic surface may first be incubated in a protein A solution and blocked with bovine serum albumin, and then incubated in an IgG solution to form antibody sensing surface. Finally, anti-IgG solution is delivered to the IgG sensing surfaces to make an estimation. The amount of anti-IgG bound with IgG immobilized by the protein A on a silicon surface is much more than that on IgG physical adsorption surface. The result confirms that the protein A can be used to immobilize antibody molecules in a highly oriented manner and maintain antibody molecular functional configuration on the silicon surface with good reproducibility

3. Conclusions

Surface modification is an important approach to improve sensing surfaces in biosensors, biochips and protein microarrays techniques. It should ideally lead to an oriented immobilization of ligands to create a sensing surface with specific bioactivity and to prevention of non-specific binding. It looks one kind of arts with an amazing balance. Weakly bound of ligands would be easily washed off from the sensing surface, causing a bioactivity decrease and instability in tests. However, a strong binding of ligands makes proteins to denature and results in a bioactivity decrease. The ligand surface concentration is a key factor also. If it is too low, it will result in low sensitivity and a narrow dynamic range of test, and if it is too large, it will lead to a high steric hindrance among ligand molecules and sensitivity decrease. Another feature for surface modification is its ability to prevent non-specific binding. In practice, we should be skillful enough to control a trade-off for the balance. The surface modification approaches on silicon reviewed above not only contribute to BIE applications in the biomedical and clinical fields, but also impart similar approaches could be implemented in other techniques with biosensing surfaces on silicon, glass and other solid substrates.

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