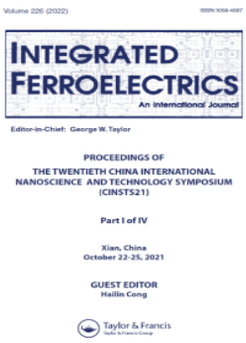

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Comparison of Random and Site-Directed Immobilization of Antibody for α-Fetoprotein Direct Immunoassay by Protein Chip

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ABSTRACT

For immunoassay, immobilization of antibody is the most important technique in bioengineering for maintaining its intrinsic activity of antigen binding. The sugar chain in the Fc region carried by antibody can be oxidized into aldehyde group by sodium *m*-periodate, which can couple with the amino-group on chemistry modified substrate surface through covalent linking. Thereby, in situ site-directed immobilization of the oxidized antibody for only one-step reaction can be performed. Here we propose a facile method that oxidized anti-human α-fetoprotein antibody (anti-AFP) was in situ site-directed immobilized on APTES treated silicon substrate utilizing microfluidic channel system for AFP immunoassay. The shape of the immobilized anti-AFP was observed by AFM and imaged by imaging ellipsometry of self-developed protein system. Results shown that the amount of antibody immobilization amount and capacity of antigen binding of random immobilization and site-directed immobilization were separately enhanced 16.0% and 7.0%. the suggested strategy has the advantages of simple, environment-friend and convenient-operation.

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protein chip; α-fetoprotein; site-directed immobilization of antibody

1. Introduction

The focus of research in life field is now shifting from genome analysis to proteome analysis that is mainly associated with the functional identification [1]. Therefore, various substrate-based techniques especially for immunoassay of antibody-antigen based reaction have been heavily developed. However, the main obstacle to be faced for these techniques is to develop effective method of immobilization of antibody onto the substrate that may preserve their native structures and biological functions. In traditional methods, the most convenient method for antibodies immobilization would be physical adsorption due to its simple and convenient operation but it is not a perfect one because antibodies are usually adsorbed on the substrate surface with random orientation leading to conformational change so that the functional sites inaccessible for antigen [2]. Furthermore, the assemble density of antibody within limited space is not

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controllable. Therefore, covalent binding of antibody through coupling reagents is alternative method that using a linker-containing substrate to form covalent linkage between the ε-amino group of antibodies and the functional group of substrate surface is usually adopted [3], but structure damage of antibody is still inevitable. For these mentioned reasons, site-directed immobilization of antibody, which can improve capacity of antigen binding, is frequently performed. The most used strategies include Protein A system, biotin-avidin system and oxidization of Fc fragment of antibody. For example, Wang et al [4] utilize Protein A for orientation-controlled immobilization of antibodies through nanometer-sized gold nanoparticles and an amine-terminated plasma-polymerized film. But this strategy is imperfect for introduce of nospecific adsorption because the protein A can combine unconcerned proteins without selectivity [5]. Cui et al [6] make use of avidin-labeled antibody to construct protein multilayers by layer-by-layer assembling technology. The shortcomings of this method are requirement of experienced operators and stringent conditions. Hoffman et al [7] oxidize the carbohydrate of antibody Fc fragment into aldehyde for linkage with the hydrazide derivatized solid support. This method requires subsequent remove of residues so it brought sophisticated treatments of purification. This study aims to compare the random and site-directed immobilization of antibody for fetoprotein immunoassay by free-label analysis and to testify the possibility of the proposed strategy of direct measurement of α-fetoprotein.

2. Experimental

2.1. Apparatus and Materials

Polished silicon wafers were purchased from General Research Institute for Nonferrous Metals (China). Rat anti-α-fetoprotein (AFP) monoclonal antibody was obtained from Biodesign. The standard AFP was bought from national institution for the control of the pharmaceutical and biological products (China). Aminopropyltriethoxysilane (APTES) was purchased from Sigma-Aldrich. Sodium hydroxide and methane acid were come from Chuandong Chemical company Inc. All other chemicals were analytical grade or better. Milli Q was used for preparations of all aqueous solutions.

2.2. Protein Chip Platform

Protein chip platform was self-developed in Institute of mechanics of CAS in China [8]. The Protein chip platform includes imaging ellipsometry system and array fabrication system. Imaging ellipsometry was used for the visualization and quantification of the protein adsorption layer on the surface of silicon substrate. The light source was a Xenon lamp, and a specific collimating system was used to provide an expanded parallel probing beam with a diameter of about 25 mm. The beam passed through a polarizer and a compensator and finally on the sample surface at an incident of 70°. An optical filter at 632.8 nm wavelength was placed in the incident optical passage to select wavelength in order to increase the ellipsometric of image. The reflected beam passed through an analyzer and an imaging lens with a spatial filter located at its focus plane, and then the ellipsometry image was focused on the sensing area of a CCD camera. The reflected light is detected with a single-channel detector such as a photomultiplier

or a photodiode. The working principle as follows when the incident light illuminate on the different areas of the same substrate (different unit dot) resulting from the same grayscale value because the different unit spot was patterned by equal antibody solution with the same concentration, after the unit dot react with AFP solution, the grayscale value will increase resulting from the formation of antigen-antibody complex. The signal in grayscale format (8 bits, 0-255 grayscale or surface density) is measured by null ellipsometry which is based on an instrument where the polarizing elements are rotated until the signal at the detector is zero (null) and the net grayscale value can be calculated whereas the grayscale value of the control was not changed. A digital image was grabbed by and stored in a computer for further evaluation by image-processing software of the program.

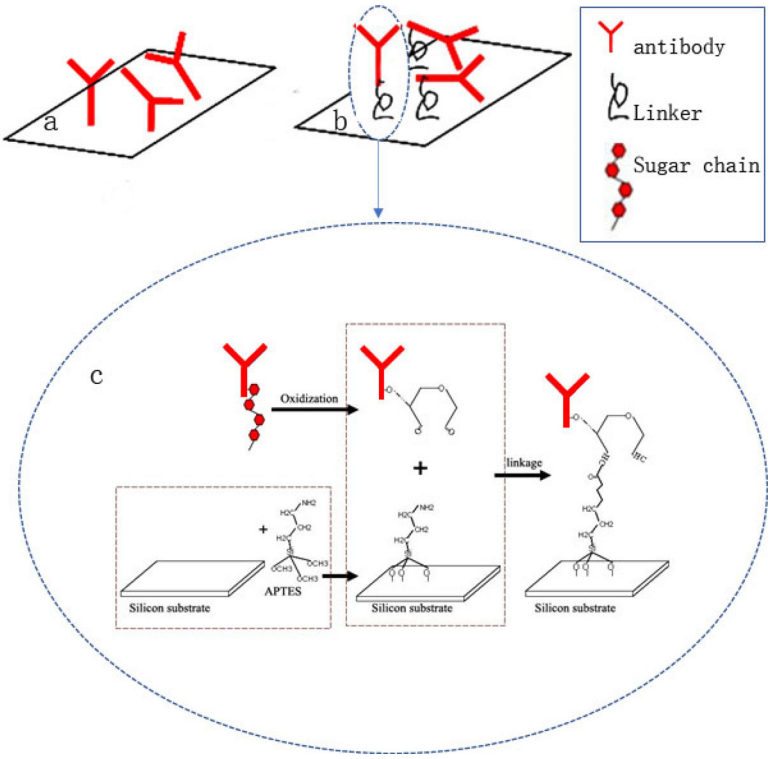
The array fabrication system was used to manufacture protein array, which was composed of sample delivery element and protein array reactor element. The sample delivery element includes a multiple micro-fluidic channel dispensing pump that can convey sample solutions by multiple parallel channels using the Teflon tubes connection under the conditions that the negative pressure was generated. Antibody solution was patterned on the substrate to form different unit dot in array format, each dot may function as capture layer. By this way, the optical biosensor can effectuate high through-put detection by multiple protein dots on the same substrate with a single experiment.

2.3. Silicon Treatment

The silicon wafers were immersed into the solution (30% H_2O_2:98%H_2SO_4=1:3 v/v) to oxidize 30 min. After rinsed by deionized water and ethanol, the washed silicon wafers were incubated in a mixture APTES and ethanol (1:4, v/v) for 2 h. Later, the silicon wafers were rinsed by ethanol 3 times and phosphate buffered saline (PBS) three times and were designated as amino-terminated substrate surface. The silicon wafers were placed into a mixture of GA and PBS (1:10, v/v), shaken lightly in a shaker for 1 h, and finally, washed by PBS buffer three times and were named aldehyde-terminated substrate surface. The silicon wafer was left in a beaker with PBS buffer until use. The surface modification was characterized by contact angle measurements (Sessile drop method).

2.4. Oxidization and Immobilization of AFP

The scheme of site-directed immobilization of antibody was referred to literature [7] with a little modification and was illustrated in Scheme 1. In brief, a 3.0 mg aliquot of anti-AFP monoclonal antibody was dissolved in 1 mL 0.5 M sodium acetate buffer, pH 5.2, to which was added 1 mL 20 mM $NaIO_4$ solution. The reaction was allowed to take place in dark environment for 1 h at room temperature with gentle shaking. The oxidized anti-AFP was directly in situ delivered onto APTES modified silicon substrate to react 20 min with a flow of 1 μL/min by microchannel system. After coupling reaction, the surface was washed thoroughly by PBS for 10 min and dried by a stream of N_2. The intact anti-AFP was directly coupled onto the APTES substrate as a control. Owing to friability of antibody, the oxidization process must be gently handled under the accurate operation. AFM observation was performed using a commercial system (Nanoscope



Scheme 1. Strategy of antibody random (a), site-directed (b) immobilization and enlarged details of b (c).

IIIa, Digital Instruments, Santa Barbara) in contact mode. A 16 µm scanner was used for surface inspection. Soft cantilevers were 200 µm long with an integrated pyramidal Si_3N_4 tip with a spring constant of 0.12 N/m. Forces for all measurements were of the order of approximately 1nN or less.

2.5. Capacity of Antigen Binding

The immunoassay of AFP antigen was conducted by above mentioned protein chip system, in which an array reactor was used for fabrication of protein dots. After blocked by BSA (1.0 µg/mL) solution, AFP solutions with different concentrations flow independently through the anti-AFP attached substrate surface in array format to react 20 min with a flow rate of 1.0 µL/min. Then, PBS was used to flush the unbound antigen away for 5 min with a flow rate of 10.0 µL/min. Finally, all of the array dots were flushed by PBS and pure water several times and subsequently send to imaging ellipsometry examination after dried under a stream of nitrogen.

3. Results and Discussions

3.1. Substrate Treatment

The cleaned silicon wafers (contact angle $<5°$) oxidized by H_2SO_4/H_2O_2 solution will reacted with APTES yielding amino group with positive charges (contact angle

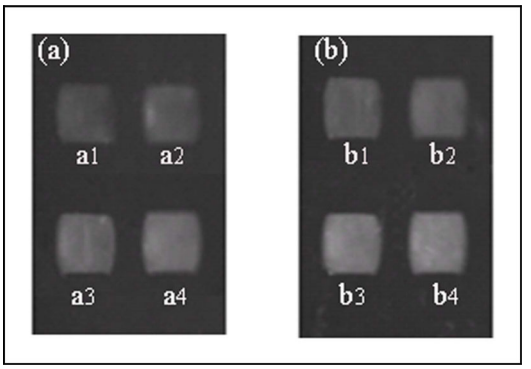


Figure 1. Ellipsometric image of protein dots on aldehyde-terminated substrate ($a_{1\sim2}$) by immobilization of anti-AFP with binding of AFP ($a_{3\sim4}$, 1.0 μg/mL) and on amino-terminated substrate ($b_{1\sim2}$) by immobilization of anti-AFP with binding of AFP ($b_{3\sim4}$, 1.0 μg/mL).

$44 \pm 1.6^{\circ}$). The latter will stepwise joint with the aldehyde group of carbohydrate chain after it was oxidized by periodic acid.

3.2. Immobilization of anti-AFP and AFM Observation

In order to understand the superiority of site-directed immobilization of anti-AFP for antigen analysis, we constructed two strategies to testify the proposed method. One is that anti-AFP was covalently linked with the as-prepared aldehyde surface and another one is oxidized anti-AFP was covalently coupled with the APTES surface for AFP determination. We then perform the ellipsometry imaging of the immobilization of anti-AFP and combination of AFP. Figure 1 shows the typical ellipsometry image of protein dots on aldehyde surface ($a_{1\sim2}$) by immobilization of anti-AFP with binding of AFP ($a_{3\sim4}$, 1.0 μg/mL) and on APTES-terminated surface($b_{1\sim2}$) by immobilization of anti-AFP with binding of AFP ($b_{3\sim4}$, 1.0 μg/mL). Every two dots of protein concentration are double for duplicate validation. Their gray values were averagely calculated. The mean grayscale value of anti-AFP immobilization of aldehyde-terminated surface is 86.0, while it rises to 115.0 after application of 1.0 μg/mL AFP. Alternatively, the mean grayscale value of anti-AFP site-directed immobilization of amino-terminated substrate is 96.0 and it rises to 128.0 after application of 1.0 μg/mL AFP. This indicates that the both the amount of oxidized anti-AFP and the amount of combined AFP on amino-terminated substrate are more than that on aldehyde-terminated surface. It was presumed that the distribution of oxidized anti-AFP on amino-terminated substrate of Fab fragment directed out the surface with higher density and the intact anti-AFP by random interspersing on aldehyde-terminated substrate with lower density.

In order to plenarily explore the detailed microscopy status of site-directed immobilization of anti-AFP, we delivered the protein dots for AFM examination. AFM has been extensively used to image biological system such as bacterial, virus, and interaction of antigen-antibody [9]. We scanned the silicon wafer APTES surface (-NH_2 surface) and revealed a highly flat and smooth surface with an average roughness less than 0.5 nm, indicating that the surface will present features if the antibody was patterned on it. Figure 2 shows AFM images obtained APTES modification and after incubation

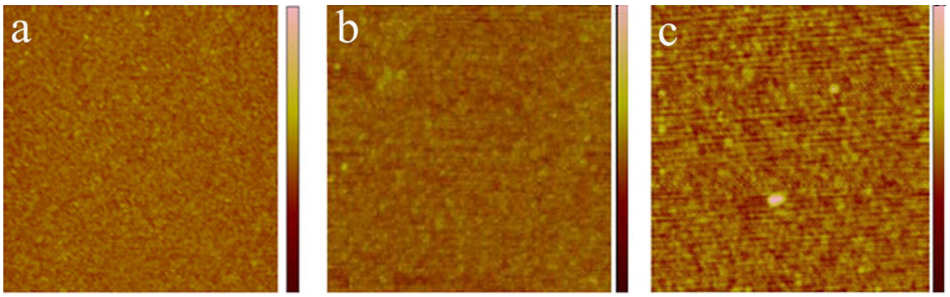


Figure 2. AFM images of aldehyde-terminated substrate (a), anti-AFP (100 μg/mL) on the modified surface by randomly (b) and site-directed (c) immobilization for 5 min.

100.0 μg/mL anti-AFP solution. It can be seen from the image that the antibody molecules were adsorbed in the form of clusters instead of disperse location. The height of these clusters was 3.0-3.3 nm varied from 20.0 to 50.0 nm in lateral size, indicating the formation of nonuniform surface aggregates. The antibody were aggregate into cluster on APTES surface when blew by a stream of N_2 observed by AFM because of the weak forces of interaction [10]. Conversely, the strong combination on amino-terminated substrate made the interaction firmly and the antibodies will not aggregate into clusters. Here, the antibody spread homogeneously on the substrate surface confirmed by AFM observation. These observations validated that there are relative strong interactions between antibody molecular and the APTES substrate after anti-AFP oxidation. It was deduced that the carbohydrate chain of the Fc region can be oxidized into aldehyde group to form imine linkage with lysine group in antibody. This linkage fasten antibody stably on the surface and the Fab fragment of the antibody molecular will direct outside of the surface and favors antigen binding.

3.3. Capacity of Antigen Binding

Standard AFP was diluted to different concentration from 10.0 μg/mL to 1.0 ng/mL, and then reacted with anti-AFP for 30 min with a flow of 1.0 μL/min. With formation of anti-AFP and antigen complex, the thickness of protein layer on the substrate surface changes, it also can be expressed as the grayscale value changes measured by imagine ellipsometry. The different protein dots on the array with different grayscale value could be automatically captured and processed in digital images. For comparison, the above manipulation was also fulfilled on aldehyde-terminated surface and APTES surface. The grayscale value plotted to logarithm of AFP concentration for both instances can be regressed and represented by the equation $Y = 48 + 8C_{AFP}$ (R^2=0.97) and $Y = 49.8 + 7.688C_{AFP}$ (R^2=0.98, Fig. 3). According calculation, the antibody immobilized amount (100 μg/ml anti-AFP was used.) and antigen combining amount (1.0 μg/mL AFP was used.) for site-directed immobilization on aldehyde-terminated surface were enhanced by 16.0% and 7.0% compared with the random immobilization on aldehyde-terminated surface. Therefore, the site-directed immobilization of antibody enables only a little improvement of antibody but more antigen binding. We yet emphasize this strategy as it can simplify the test steps for its ability of in situ site-directed immobilization of antibody with simple operation and is suitable for quick test on spot in practice.

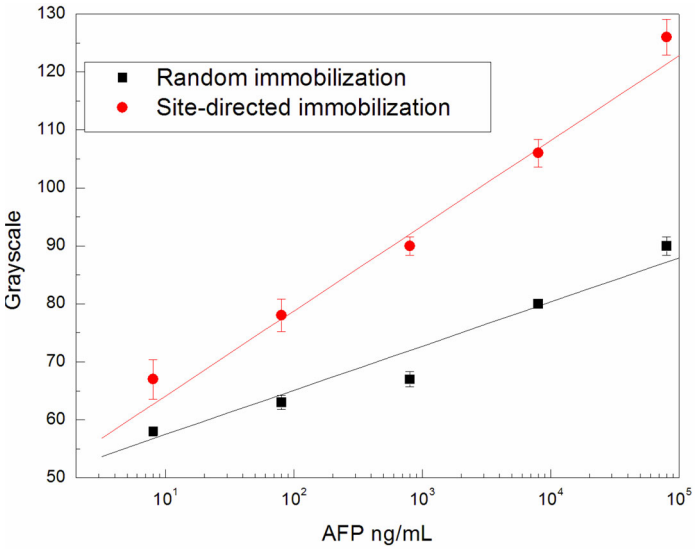


Figure 3. Typical dose-response curves obtained by serial diluted AFP to react with the site-directed immobilized antibody (●) on APTES surface and random immobilized antibody (■) on aldehyde surface.

4. Conclusions

The carbohydrate chain of anti-AFP can be oxidized into aldehyde group and in situ covalently linked with the APTES treated substrate, which was confirmed by AFM observation. This makes the Fab fragment of the antibody molecular directing outside the substrate surface and favors capacity of antigen binding. The amount of antibody immobilization together with capacity of antigen binding for both random immobilization and site-directed immobilization were separately enhanced 16.0% and 7.0%. The site-directed immobilization of antibody can be exploited for application of protein chip as it can simplify the operation steps for its ability of in situ immobilization of antibody.

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