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Orientation of Antibodies on a 3-Aminopropyltriethoxylsilane-Modified Silicon Wafer Surface

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Abstract. An antibody can be specifically oxidized with periodate ($NaIO_4$) on the carbohydrate side chains at its C-terminal. Rabbit anti-hepatitis B surface antigen (anti-HBsAg) IgG antibodies were bound to the silicon wafer surface by covalent bonds between aldehydes generated on the carbohydrate side chains of the antibodies and the reactive amine groups of 3-aminopropyltriethoxylsilane(APTES)-modified silicon wafer surfaces. A control experiment was also performed by direct attachment of antibodies to glutaraldehyde-treated silicon surfaces. Two different coupling antibody strategies were investigated in this paper. Atomic force microscopy was used to observe the orientation of the site-directed and random attachment of rabbit anti-HBsAg IgG antibodies and the conservation of their antigen-binding capacity (AgBC) was assessed using an enzyme immunoassay (EIA).

Key words: antibodies, antigen-binding capacity

1. Introduction

The immobilization of antibodies to solid surfaces is important in biotechnology, diagnostics, and sensing applications [1–4]. When antibodies are immobilized onto solid surfaces, a lower antigen-binding capacity (AgBC) often ensues due to the improper orientation of the antibody [5]. The preservation of AgBC on surfaces by controlling antibody orientation has become an important consideration in applications in which antibody immobilization is required. However, although associated with some disadvantages, most conjugation procedures couple antibodies to solid supports via the ϵ-amino groups of one or more of their lysine residues [3–4, 6]. Using this immobilization strategy, the potential for multiple attachments, with resulting conformational distortions, is high. More problematical yet is the likelihood of an attachment at readily accessible and reactive amino terminals, which are

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the sites of antigen binding. In either case, a reduction in the Ab's specific ability to bind antigen is the likely outcome of a random coupling to available amines. To avoid this dilemma, researchers have been attempting to use an attachment chemistry which assures coupling to a site far removed from the antigen binding sites. One approach to achieving this goal is to perform the coupling via the carbohydrate moieties located in the antibody molecule's fragment crystallizable (Fc) region, close to the "hinge" between this C-terminal domain and the two N-terminal fragment antigen binding (Fab) domains. This requires an oxidation of the antibody to produce aldehyde groups which can then be used to link the molecules to primary amines or hydrazides on the surface [7–9]. No system is available that permits the binding site of the antibody and the structure of the interface to be controlled in detail sufficient for investigation of the orientation of immobilized antibodies at the molecular level on solid surfaces [10–11]. The present contribution develops sequentially a "model" system for the site-directed immobilization of protein. The oxidation of antibody carbohydrate residues by periodate is a versatile approach for the modification of antibodies [7–9], which are attached to drugs and labels or chromatography supports. Surface derivation of a silicon wafer with 3-aminopropyltriethoxylsilane (APTES) in dry toluene resulted in covalently bonded siloxane films with surface coverage that was relatively controllable by regulating the reaction conditions [12]. A simple, convenient and efficient method for site-directed incorporation of aldehydes generated on the carbohydrate side chains at the C-terminal of IgG using periodate-oxidized reactions is explored as a means of ensuring site-directed immobilization of IgG on the reactive amino groups of the APTES modified silicon wafer surface. The site-directed immobilizing strategy used in this paper allowed control over the composition of the interface and maintained the AgBC of the attached antibodies. Each step of the derivatization of a silicon substrate by APTES, and the subsequent site-directed and random immobilization of antibodies, was characterized with atomic force microscopy (AFM) and enzyme immunoassay (EIA).

2. Materials and Methods

2.1. AMINATION OF SILICON WAFER SURFACES

Single crystal silicon wafers (n-type, $4 \times 4 \times 1$ mm) were from the microelectronics center of Southeast University, China and cleaned with "Piranha solution", a 30:70 mixture of 30% hydrogen peroxide (H_2O_2) and concentrated sulfuric acid (H_2SO_4) by supersonic wave for 1h and then thoroughly rinsed in Milli-Q grade deionized (DI) water, absolute ethanol and DI water by supersonic wave respectively. A solution of 30 ml dry toluene containing 0.5 ml (2.15 mmol) 3-aminopropyltriethoxysilane (APTES) from Sigma was ready for silanization. Amine group derivatization of silicon wafers was performed by incubating single substrate samples at 120 °C for 3 h in the above solution and further incubation for another 1 h at room temperature, before ultrasonically washing with chloroform

and absolute ethanol. The APTES-modified substrates were dried in nitrogen for AFM measurements and stored in water or organic solvents for further reactions.

2.2. PRODUCTION AND PURIFICATION OF RABBIT ANTI-HBSAG ANTIBODIES

Rabbit anti-HBsAg polyclonal antibodies were used as a model system in these studies. The rabbit anti-HBsAg serum was prepared in our laboratory and HBsAg standard samples were obtained from the Center for Clinical Laboratory Science, Jiangsu Province. The rabbit anti-HBsAg fraction was prepared by precipitation in 50% saturated ammonium sulfate followed by anion exchange chromatography (DEAE-cellulose, Pharmacia). Protein A chromatography was used as the final purification step.

2.3. OXIDATION OF ANTIBODIES

A 3 mg aliquot of the rabbit anti-HBsAg was dissolved in 1 ml of 0.15 M sodium acetate buffer, pH 5.2, to which was added 1 ml of a 50 mM solution of $NaIO_4$. The reaction was allowed to take place for 1 h at room temperature with shaking. Unreacted $NaIO_4$ was then separated from the reaction mixture by dialysis (8,000–10,000 MW cut-off). These samples were dialyzed at 4 °C for 2 h against one 2.0-liter portion of pH 5.2, 20 mM acetate buffer containing 0.15 M sodium chloride. This was followed by three additional dialysis cycles, each performed for a minimum of 2 h.

2.4. IMMOBILIZATION OF ANTIBODY

Two types of immobilization chemistry were employed for the Rabbit IgG: (1) direct attachment of antibodies (0.35 ml of a 1 mg/ml solution in PBS buffer, pH 7.2) to glutaraldehyde-treated silicon wafer surfaces in carbonate buffer (CB) of pH 9.2 (7.5 ml added to the antibody solution) for 6h at room temperature; (2) Site-directed immobilization of oxidized IgG (1 mg/ml) to APTES derived silicon wafer surfaces in acetate buffer of pH 5.2 containing 5 mM $NaBH_4$ for 12 h at 4 °C. After either of the coupling reactions, the surface was thoroughly rinsed in PBS buffer containing 0.02% Tween 20.

2.5. ATOMIC FORCE MICROSCOPY

AFM measurements were performed using a commercial system (Nanoscope IIIa, Digital Instruments, Santa Barbara) in the 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. Typical forces for all measurements were of the order of approximately 1 nN or less.

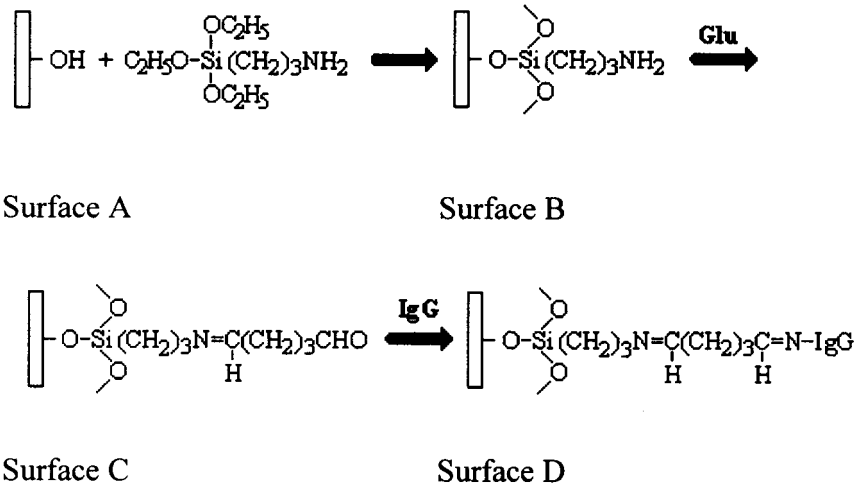
2.6. IMMUNOASSAY

The presence of the immobilized IgG molecules and the conservation of their biological structures were assessed using enzyme immunoassay (EIA). Following rabbit anti-HBsAg immobilization, silicon wafer samples ($4 \times 4 \times 1$ mm) were placed in individual compartments of a multiwell plate preincubated overnight at 4 °C with 2% bovine serum albumin (BSA) in PBS to block nonspecific adsorption, and then the samples were covered with 200 μl of PBS containing BSA for 2 h at 37 °C and shaken constantly after transfer to the well to prevent artifacts produced by adsorption of enzyme-conjugated IgG in the next step. The BSA solution was removed and the compartments were rinsed with 250 μl of 0.02% Tween 20 in PBS ($\times 2$) and 250 μl of distilled water ($\times 2$). 100 μl of 1 ng/ml standard HBsAg sample in PBS was added in individual compartments for 60 min at room temperature. The HBsAg solution was removed and the compartments were rinsed with 250 μl of 0.02% Tween 20 in PBS (($\times 2$) and 250 μl of distilled water ($\times 2$). Horseradish peroxidase (HRP) was attached to polyclonnal goat IgG using a standard procedure. 100 μl portions of one of the commercial HRP-IgG solutions were then added to each well. Excess HRP-IgG was removed after 60 min incubation at 37 °C and each well was washed with 250 μl of 150 mM NaCI, followed by 0.02% Tween 20 ($\times$) and 200 μl of water ($\times 3$). Subsequently, a drop of solution A (TNB, 50 μl) and a drop of solution B (H_2O_2, 50 μl) from a standard EIA kit for the determination of HBsAg (Shanghai SIIC Kehua Biotech CO., LTD, China) were added to each well respectively, and incubated for 1 minute at 37 °C. Finally, a drop of ending solution (50 μl) was added to each well to stop the reaction. The absorbance (λ = 450 nm) of aliquots of 150 μl from each well was measured (CliniBIO 128, Austria).

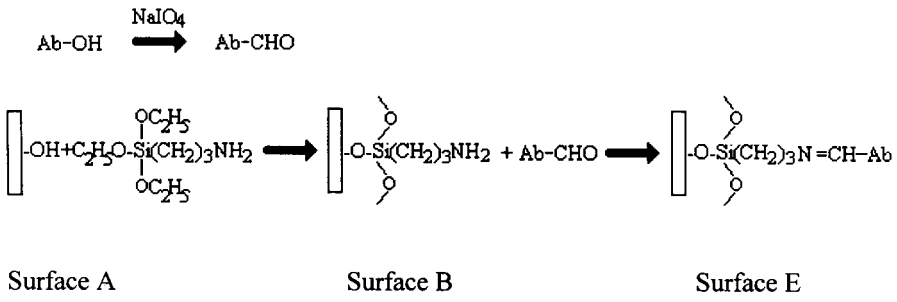
3. Results and Discussions

Scheme 1 shows the direct attachment steps of antibodies to glutaraldehyde-treated silicon wafer surfaces: first, silanization of a cleaned silicon wafer (Surface A) with APTES in toluene provided surface B with terminal reactive amino groups, and then reaction of surface B with glutaraldehyde yielded surface C with terminal reactive aldhyde groups, which were subsequently reacted with rabbit IgG to give random antibody attachment (surface D). Scheme 2 shows the site-directed immobilization steps of oxidized antibodies to APTES-modified silicon wafer surfaces. In the first step, silanization of a cleaned silicon wafer (Surface A) with APTES in toluene gave surface B with terminal reactive amino groups, which are then directly reacted with oxidized rabbit IgGs, resulting in site-directed orientation of oxidized antibodies (surface E).

The derivatization of a silicon wafer surface is conveniently begun by a silanization procedure which introduces amine groups onto the surface. This is commonly done by a reaction of carefully cleaned silicon surfaces with APTES [13–14]. It is thought that the hydrolyzable ethoxy groups of 3-aminopropyltriethoxysilane condense with the surface silanols, releasing ethanol and leaving the amino end



Scheme 1. Random attachment procedures of antibodies.



Scheme 2. Site-directed immobilization steps of antibodies.

group free. The free amino group is then capable of coupling to the aldehyde groups of glutaraldehyde and other compounds. For attachment to glass, silicon, or metal oxides for biosensor applications, short-chain amino silanes have been used. However, the short chain silane linkage is susceptible to hydrolysis and instability of protein layers [15]. In our experiments, we obtained a well-oriented layer of amino groups using surface derivation of silicon wafer with APTES in dry toluene at 120 °C for 3 h followed by 1 h incubation at room temperature, with 75% of the amino groups oriented outward from the surface and 25% oriented toward the surface, as confirmed by XPS [16]. The thickness of the organic siloxane layer and the number of reactive NH_2 groups introduced by the above silanization method with APTES were characterized by Xiao *et al.* using ellipsometry, and ^{14}C-formaldehyde radiology measurement [12]. The silanization process led to mutilayer formation with a thickness of 5.2 nm and a surface coverage of 1.54 nmol NH_2–groups per cm^2. Figures 1a–b shows the representative images of the untreated silicon wafer surface and the covalently bonded siloxane film observed routinely using the AFM respectively (surface A and surface B). The APTES derivatized silicon wafer

Table I. Assays for the presence and specific antigen-binding capacity of randomly attached and site-directly immobilized rabbit anti-HBsAg antibodies.

Treatment of the surfaces	EIA[a]
APTES-modified surface (surface B)	0.01
Random attachment (surface D)	0.16 ± 0.02
Site-directed immobilization (surface E)	0.32 ± 0.02

[a] Results in absorbance units, the result of enzymatic activity of the immunocomplex on the surface D and Surface E.

surface rinsed with different rinsing solutions appears more stable and dense. We found that many APTES domain features homogeneously spread over the silicon wafer surfaces. This is likely to be the result of increasing siloxane film lateral polymerization and network formation.

Figure 2 shows a schematic representation of the structures of siloxane film networks (top), of random orientation of antibodies (middle), and of site-directed orientation of antibodies (bottom). From crystallographic data it is known that an IgG antibody (MW 146 000) is arranged in three discrete domains; specifically two Fab fragments and one Fc [17–19]. An estimate of the dimensions as would be observed by AFM can be made from X-ray crystallographic works on isolated Fab fragments [20] and transmission electron microscopy images (TEM) [21–22], giving an expected upper limit to the molecular dimension on the surface of approximately 16 to 19 nm.

Figure 3 shows the topology of orientation of randomly attached IgG antibodies on an APTES film. No individual antibodies could be clearly identified. Larger features probably correspond to the random packing of the aggregate of IgG and these could also be denatured sites.

The AFM images in Figure 4 are typical scans recorded from oxidized IgG antibodies site-directly immobilized onto surface B by their carbohydrate side chains. The images show not only that some Y-like structure features are observed to form surface patterns but also that their conformations appear more stable and dense when bound on the silicon wafer surface. Such images could be obtained reproducibly with no evidence of severe molecular distortion at the imaging force which were employed. Visual inspection of data suggests that these Y-like structure features range in dimensions from approximately 30 to 40 nm, and they are proposed to result from the IgG antibodies, although these values are somewhat larger than would be expected. As in the IgG data there is some increase in observed molecular dimensions compared to previously published crystallographic data [21–22], which was proposed to principally result from the effects of a finite probe size and not from deformation of the molecular species due to the imaging forces employed [23].

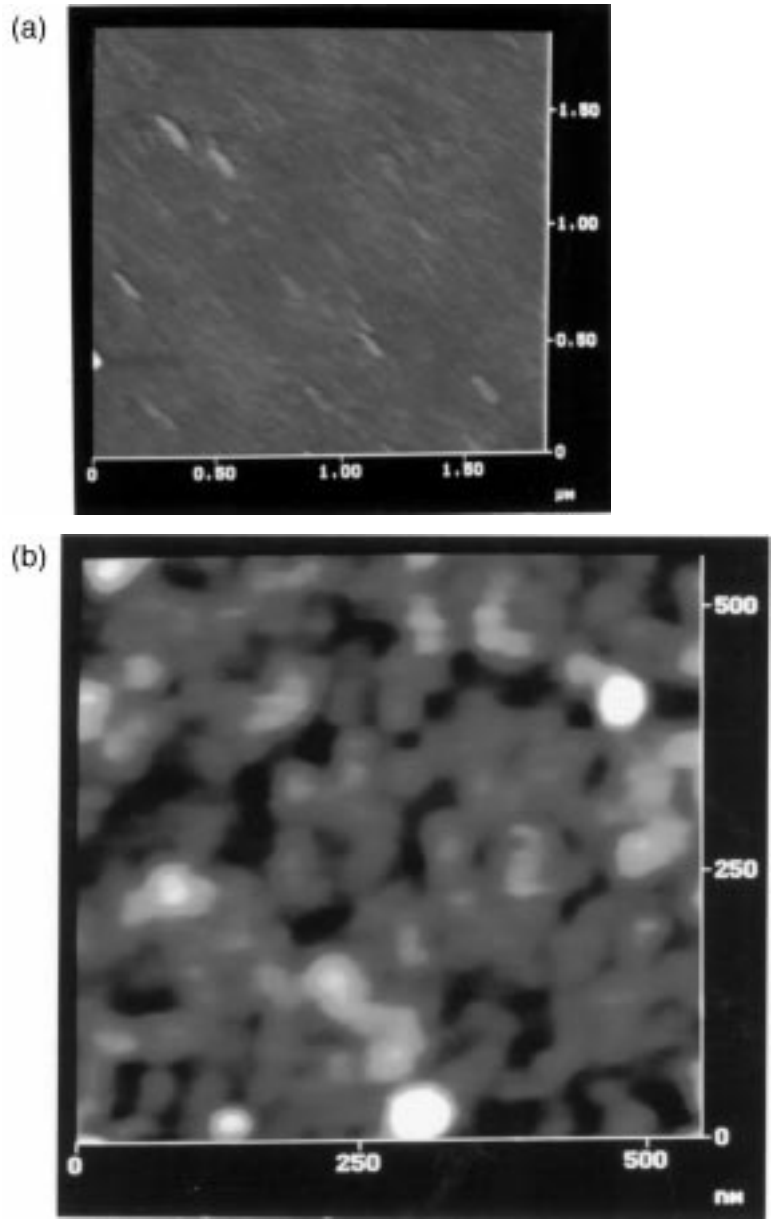


Figure 1. The AFM images of (a) Si-surface before APTES incubations at 1.50 × 1.50 μm and (b) the APTES siloxane film at 500 × 500 nm.

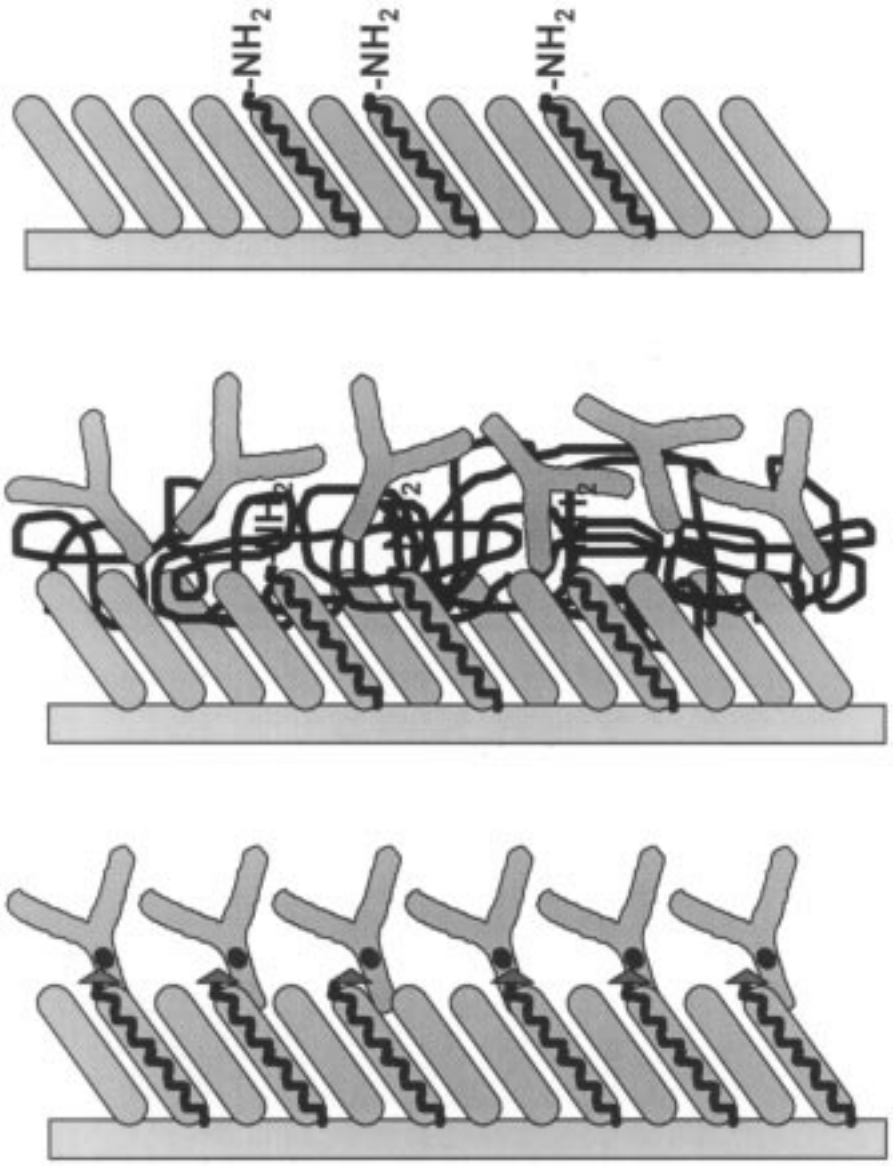


Figure 2. The Schematic representation of the structures of siloxane film networks (top), of random orientation of antibodies (middle), and of site-directed orientation of antibodies (bottom).

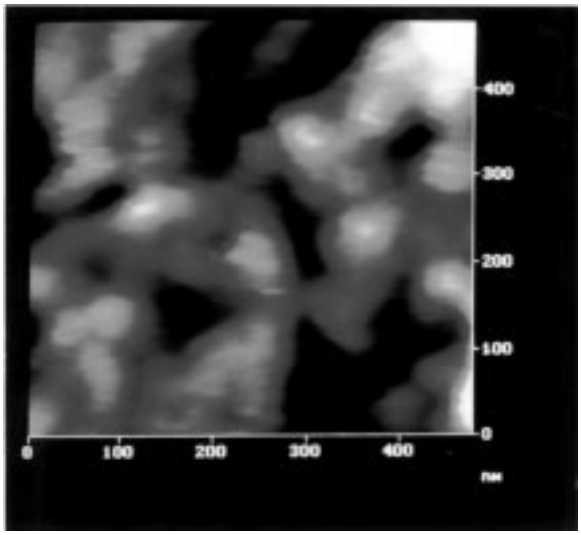


Figure 3. The AFM image of randomly oriented IgG molecules onto the APTES film at 500 × 500 nm.

The conservation of AgBC is always a very important criterion for the presence of antibodies onto solid surfaces, which is usually assessed by EIA. Table 1 shows EIA results of the absorbance (λ = 450 nm) of randomly attached and site-directly immobilized IgG samples. It is found that the AgBC of the site-directly immobilized IgG sample per 16 mm^2 area is twice that of the randomly attached IgG sample.

4. Conclusion

This paper demonstrates that APTES-treated silicon wafer surfaces are capable of covalently attaching site-directed antibodies in a dense, homogeneous siloxane film. Site-directed orientation of antibodies to silicon wafer surfaces can be performed through coupling via the carbohydrate moieties in their hinge regions. The method emphasizes the utility of siloxane film networks to create and control the properties of surfaces in a well-characterized and useful manner that allow the in-depth study of a complex problem: the site-directed immobilization of proteins at solid surfaces without loss of their function.

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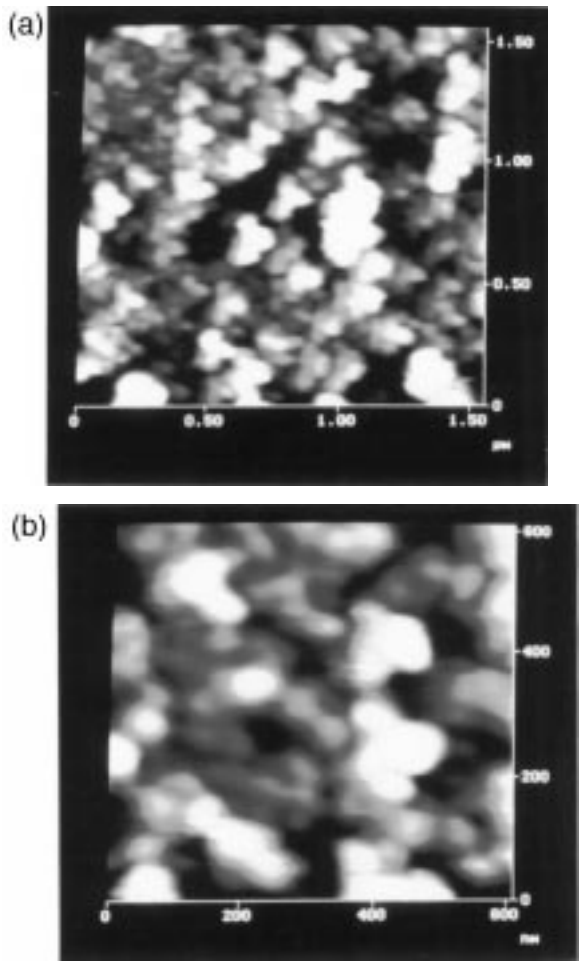

Figure 4. The topology of orientation of site-directly immobilized antibodies on the APTES film on silicon wafer surface: (a) 1.50 × 1.50 μm; (b) 600 × 600 nm.

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