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Two methods for glass surface modification and their application in protein immobilization

Ming Qin [a], Sen Hou [a], LiKai Wang [a], XiZeng Feng [a,*], Rui Wang [b], YanLian Yang [b], Chen Wang [b,*], Lei Yu [c], Bin Shao [c], MingQiang Qiao [c,*]

[a] *The Key Laboratory of Bioactive Materials, Ministry of Education, College of Life Science, Nankai University, Tianjin 300071, PR China*
[b] *National Center for Nanoscience and Technology, Beijing 100080, PR China*
[c] *Institute of Molecular Biology, College of Life Science, Nankai University, Tianjin 300071, PR China*

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Abstract

Protein immobilization is a crucial step in protein chip, biosensor, etc. Here, two methods to immobilize proteins on glass surface were analyzed, one is silanization method using 3-aminopropyltriethoxysilane (APTES), and the other is hydrophobin HFBI coating. The modified glass surfaces were characterized with X-ray photoelectron spectroscopy (XPS), water contact angle measurement (WCA) and immunoassay. The results of XPS and WCA illustrated that the surface property of glass can be changed by both the two methods. The following immunoassay using microcontact printing (μCP) verified that both methods could help protein immobilization effectively on glass slides. Compared with the amine treatment, it is concluded that hydrophobin self-assemblies is a simple and generic way for protein immobilization on glass slides, which has potential application in protein chips and biosensors.

Keywords: Hydrophobin; HFBI; Protein adsorption; Glass; Surface modification

1. Introduction

With the development of protein chips and biosensors on solid supports, the demand for the protein immobilization is increasing rapidly. Protein immobilization, to a great extent, depends on the modification of substrate surface. Since it can resist high temperature and rigorous rinse, consume fewer samples and keep lower noise signals of fluorescence, glass has been one of the widely used substrates for protein chips [1–3]. Hence, many methods have been reported to modify glass slides [4,5], including poly-L-lysine coating [6], silanized surfaces [7], low molecular weight cross-linkers [8], protein-coated surfaces [9], avidin-functionalized surfaces [10], self-assembled monolayer (SAM) [11], polymer coating [12], and affinity tags for protein immobilization (e.g., arginine tags and histidine tags) [13,14]. The binding mechanisms between the modified surface and the protein include physical adsorption and covalent binding [4]. However, several problems need to be solved. For example, due to the surface hydrophobicity, one problem caused by silanized surfaces is protein denaturation. In this paper, two methods for protein immobilization on glass slides were analyzed, one is the traditional method of silanization [7]; the other is the novel one with HFBI coating, by which enzymes could be immobilized on solid surface recently [15]. The aim of this work is to compare the two methods of surface modification and study the feasibility of HFBI coating as a linking layer for protein immobilization on glass surfaces.

Silanization is the most common way to introduce a variety of functional groups onto glass surface [16]. Originated from DNA microarray, amino or aldehyde-containing silane treatments could also suit to protein assays, in which alkylsilanes bind covalently by nucleophilic attack of surface hydroxyl groups on silicon atoms. One of the differences between the two methods is the binding mechanism, since amine surfaces could only adsorb proteins via electrostatic interactions, while aldehyde surface could covalently bind the protein through Schiff base reaction. Another difference is that the former is slightly hydrophilic, while the latter is hydrophobic. Both of them could

* Corresponding author. Tel.: +86 22 2350 7022; fax: +86 22 2350 7022.
E-mail address: xzfeng@nankai.edu.cn (X. Feng).

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receive excellent assay sensitivity and low background noise [4]. What is more, amine surface treated with silane reagents, such as 3-aminopropyltriethoxysilane (APTES), could be changed to aldehyde surface by exposing to glutaraldehyde.

Recently, hydrophobin coating has been studied as media to immobilize proteins on solid supports. Hydrophobins, first purified in filamentous fungi [17,18], are amphiphilic molecules and keep strong surface activity. Based on this property, many aspects of fungal development have been related with hydrophobins [19–21]. For example, they coat aerial structures and make them hydrophobic, such as spores and fruiting bodies [22,23]; by self-assembly on the cell-wall-interface and reducing the water surface tension, they are involved in the escape of fungi from the aqueous environment into the air [24]. Furthermore, hydrophobins are also involved in the attachment of hyphae to hydrophobic surfaces. For example, during pathogenic interactions, hydrophobins can help them attach to their hosts, such as on insect cuticles or plant leaves [25].

Hydrophobins are fairly small (7–15 kDa) and share eight Cys residues in their primary sequence [23]. According to their hydropathy patterns and solubility characteristics, hydrophobins can be classified into classes I and II [26]. The membranes of class I members, such as SC3 from *Schizophyllum commune*, are very insoluble, which can only be dissolved in strong acids such as formic acid or trifluoroacetic acid (TFA) [27]. Moreover, they often form random patterns of nanoscopic rodlets and their bundles on surfaces [28,29]. It is reported that class I hydrophobins show an increase of β-sheet structure over insoluble state when forming membranes, which is not observed in class II hydrophobins [30,31]. Assemblies of class II hydrophobins dissociate more easily, and can be dissolved in ethanol or sodium dodecyl sulfate [32]. The class II hydrophobins HFBI and HFBII from *Trichoderma reesei* have been found to form highly ordered monolayer films and crystalline fibrils [33–35]. Besides, both class I and class II hydrophobins can interact with each other in their assemblies [36]. The recent determination of 3D-structure of HFBII and HFBI confirmed that hydrophobins are globular and amphiphilic, which help to understand the structure–function relations of other hydrophobins [37,38].

Due to the amphiphilic nature and self-assembly properties, hydrophobins have also been applied in some areas [23,39]. They can be used to improve the biocompatibility in biomaterials and biomedical applications [40,41], help hair care products survive several shampoo washes and stabilize emulsions in ointments for personal care [42], purify target protein rapidly and efficiently in a detergent-based two-phase system [43], etc. Recently, hydrophobins have also been reported to reduce the nanoscale relative surface of friction for biomedical application [44] and form protein–dendron conjugates with high affinity binding of DNA [45]. Besides, they have been applied to immobilize enzymes and prevent the protein denaturation on both hydrophobic and hydrophilic surfaces for biosensors and electrodes [15,28,46–48]. Hence, with hydrophobin coating, protein immobilization may be effectively achieved. However, hydrophobin coating on glass slides for protein adsorption has not been exclusively studied, which can be applied in the area of protein chips.

In this paper, two methods of glass surface modification were studied, in which the surfaces were modified with either silane reagent APTES or class II hydrophobin HFBI. The modified surfaces were analyzed with X-ray photoelectron spectroscopy (XPS) and water contact angle (WCA) measurements. Subsequently, the protein immobilization was carried out on the modified surfaces using microcontact printing (μCP) technology with a polydimethylsiloxane (PDMS) stamp [49]. After comparison with the traditional silanization method, it is concluded that HFBI coating is a simple and generic way for protein immobilization, and has potential application in protein microarrays and biosensors.

2. Materials and methods

PDMS elastomer kit (Sylgard 184 (Dow Corning)), curing agent, bovine serum albumin (BSA) and 3-aminopropyltriethoxysilane were purchased from Sigma. Chicken immunoglobulin (IgG) was used as antigens, and fluorescein isothiocyanate (FITC) labeled anti-chicken IgG developed in rabbit was used as its antibody. They were also purchased from Sigma. The class II hydrophobin HFBI was supported by Professor Mingqiang Qiao. Glass cover slides (Sail Brand) were obtained from Taizhou Dongsheng Glass Co. Ltd., China. Liquid detergent was purchased from Shanghai White Cat, China. All the reagents in common use, were purchased from commercial suppliers in highest purity and were used as purchased. Aqueous solutions were prepared by redistilled water.

2.1. Modification of glass with APTES

Glass preparation and cleanliness are of critical importance in the experiments. Glass cover slides were cleaned carefully with detergent (1:100 by volume), flushed thoroughly in redistilled water and ultrasonicated in water for 10 min. Then the slides were placed in freshly prepared solution of ammonia solution (25% NH_3, 30% H_2O_2 and redistilled H_2O) (1:1:5 by volume) for 3 min, rinsed with redistilled water, and immersed in a freshly prepared solution of 37% HCl, 30% H_2O_2 and redistilled H_2O (1:1:5, by volume) for 5 min. Subsequently, the slides were washed thoroughly with redistilled water, ethanol and acetone one after another. After dried in an oven at 110 °C for 1 h, the substrates were immediately modified by immersing pretreated glass slides in a solution of APTES (5% by volume) in an anhydrous toluene solution for 5 min. After rinsing with toluene and acetone for several times, the modified slides were dried in an oven at 110 °C for 1 h prior to use.

2.2. Coating of glass with HFBI

Glass cover slides were cleaned carefully with detergent (1:100 by volume), rinsed thoroughly with redistilled water, ultrasonicated in water for 10 min, immersed in 75% ethanol and then dried with a flow of pure nitrogen. Then, freshly pretreated glass sheets were incubated for 20 min at room temperature in an aqueous solution (100 μg/ml) of hydrophobin HFBI, allowing

the protein to adhere to the slides. After blowing off the excess solution, the sheets were dried with a flow of pure nitrogen. Thus, a thin film of HFBI can be formed on the slide.

2.3. X-ray photoelectron spectroscopy

The chemical compositions of bare, APTES-modified and HFBI-coated glass slides were analyzed using XPS Apparatus (PHI-5300) from PHI, USA. The experiment conditions are as follows: the energy of excitation source monochromatic Mg Kα radiation is 1253.6 eV, and the survey scan range is 0–1100 eV. The electron take off angle was fixed at 45°. After scanning the overall spectrum for 2–3 min, peaks over narrow ranges were recorded for C1s, O1s, N1s, S2p, and Si1s for 4–5 min. The energy resolution of the analyzer is 0.8 eV, and the sensitivity is 80–1600 KCPS. The peaks in the elemental core-level spectra were fitted using UNIX on an Apollo Domain series 3500.

2.4. Wettability measurement

Water contact angle measurement was carried out with a 5-μl water droplet at ambient temperature with an optical contact angle meter (Dataphysics Inc., OCA20). The reported WCA values are averages of three measurements made on different positions of the sample surface. All measurements for all surfaces were within ±2.0° of the averages.

2.5. Microcontact printing of proteins on the APTES-modified or HFBI-coated glasses [50]

2.5.1. Fabrication of PDMS stamp

The PDMS stamp for μCP was made by casting on silicon master. The liquid PDMS is polymerized in the mold which was formed by the wafers. Upon curing at 60 °C overnight, the micro-patterned master was replicated with a PDMS-to-cross-linker ratio of 10:1. After peeled off, a flexible transparent stamp is obtained, on which the structures can be as small as 100 μm [51,52].

2.5.2. Printing proteins

The same method was used to pattern antigen on both the APTES-modified and HFBI-coated glass slides. The PDMS stamp was cleaned several times with redistilled water 75% ethanol solution prior to use. Then the PDMS stamp was 'inked' in chicken IgG solution (200 μg/ml) for 2 min, and excess solution on the stamp was removed with a piece of lens paper. Then the stamp was pressed on the slides softly to make better contact between the surfaces.

Then the substrates were blocked with BSA (10 mg/ml) solution for 20 min washed thoroughly with redistilled water and dried with a flow of pure nitrogen. Subsequently, the patterned surfaces were incubated with FITC-labeled anti-chicken IgG (200 μg/ml) for a few minutes and rinsed with redistilled water. After dried with a flow of pure nitrogen, the printed stripes could be observed with inverted fluorescence microscopy (Nikon TE2000-U, CCD-Rtke, Japan). All proteins were dissolved in phosphate-buffered saline solution (PBS) at pH 7.4. The pH of redistilled water for washing was 7.4.

3. Results and discussion

The APTES-modified and HFBI-coated glass slides were characterized by XPS, WCA and immunoassay using μCP.

3.1. Characterization of substrate surfaces by X-ray photoelectron spectroscopy

The main elements of glass are carbon, oxygen and silicon. Once the glass slides were modified with APTES or HFBI, the nitrogen element from APTES or HFBI protein should be exposed outwards on the substrates, which could be detected by XPS.

The chemical composition of glass surfaces before and after modification was investigated with XPS. The full XPS spectra of the bare and modified glass slides on the same scale were shown in Fig. 1; Fig. 2 showed the XPS spectra of N1s

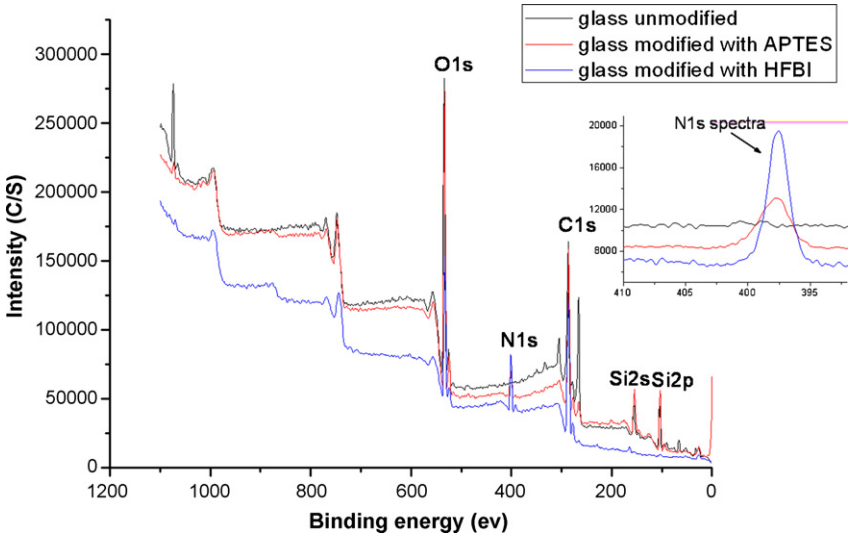

Fig. 1. Full XPS spectra of the bare, APTES-modified and HFBI-modified glass slides on the same scale.

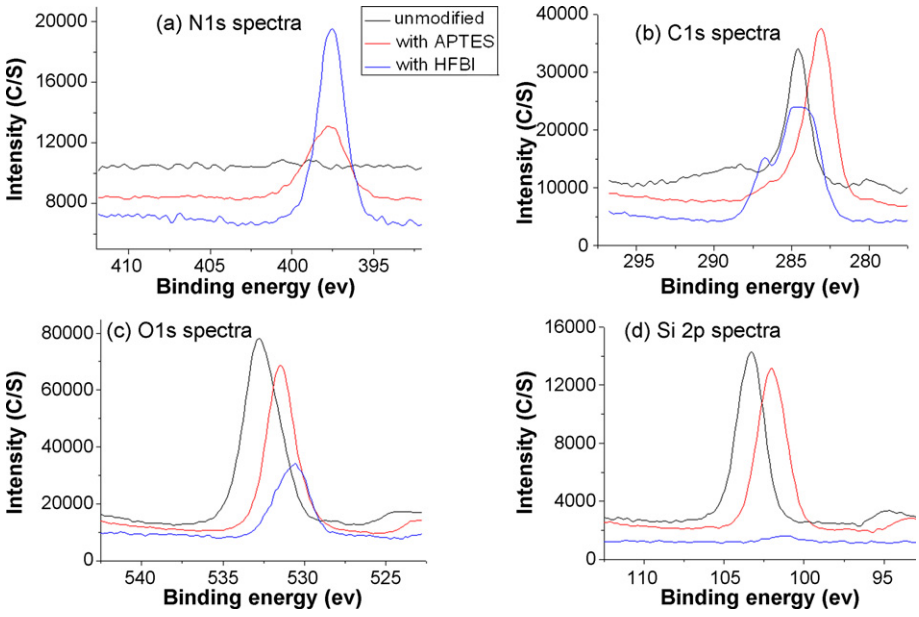

Fig. 2. XPS spectra of N1s, C1s, O1s and Si2p on the bare, APTES-modified and HFBI-modified slides on the same scale.

(400 eV), C1s (287 eV), O1s (534 eV) and Si2p (106 eV) on the bare and modified glass slides on the same scale. It could be seen in Fig. 1 that the characteristic N1s peak changed from the background noise level to the prominent peak at 400 eV with APTES modification. As shown in Fig. 2, APTES modification leads to dramatic intensity increase of N1s and C1s, while the signal of O1s and Si2s decreased. The signal increase of N1s (400 eV) peak and the C1s peak (287 eV) certified that the glass surface has been covalently linked with APTES. Similar changes also occurred on the XPS spectra of HFBI-coated glass surface, as shown in Figs. 1 and 2. Only one difference between the two kinds of modified surfaces on the spectra is that the HFBI protein coating often caused more dramatic changes on the peak intensity. For example, on the protein-coated surface, the increase of N1s peak intensity is higher, and the Si2p peak even disappeared from a prominent peak, as shown in Table 1. We think that two reasons may be responsible for the difference. Firstly, the chemical composition of HFBI protein is much more different from that of glass, seen in Table 1; secondly, hydrophobin HFBI could form a film hiding the substrate to a great extent, while reactive amine group in APTES only partly bind on the substrate covalently, supported by the change of the element Si value before and after modification (in Table 1).

Table 1
The relative atomic compositions on the bare glass, APTES, HFBI, APTES-modified glass and HFBI-modified glass

Sample type	C (%)	N (%)	O (%)	Si (%)
Bare glass	25.20	0.20	54.91	19.69
APTES	48.87	6.33	21.72	12.67
APTES-modified glass	42.40	6.32	34.04	17.23
HFBI	50.71	16.90	21.79	0
HFBI-modified glass	61.86	13.15	22.74	0.91

3.2. Water contact angle

Generally, after surface modification the changes of surface chemical composition occurred with the change of surface wettability on the same time. Surface wettability, analyzed with WCA, could verify the effect of modification indirectly. The static water contact angles of bare and modified glass slides were shown in Table 2. The WCA of bare glass slides is 25.7°, which shows that the glass surface is moderate hydrophilic. The WCA of APTES-modified glass is 59.0°, which means that silanization treatment could slightly decrease the wettability of glass surface; while the HFBI coating could lower the WCA of glass slides to 16.8°. With the data of WCA, we can include that the properties of glass surfaces have been surely changed by both two methods of modification. Besides, the WCA values of modified surfaces were not changed even after storing for several days.

The changes of WCA before and after modification were related with the chemical property of APTES or HFBI. The hydrophobic alkane group in APTES may result in the increase of WCA, which is consistent with previous report [53]. The adsorption of HFBI on solid supports is related with its amphiphilic property. Yet, our finding that the WCA of HFBI coating surface decreased is opposite to former reports on hydrophobins, which indicated that the hydrophobin-modified glass surface become slightly hydrophobic [18,23,54]. We sup-

Table 2
Water contact angle[a] of bare, APTES-modified and HFBI-modified glass surfaces

Sample type	Bare glass	APTES-modified glass	HFBI-modified glass
WCA	25.7°	59.0°	16.8°

[a] $n = 3$. The WCA values were averaged from three measurements at different locations.

pose a hypothesis to explain these seeming contradicting finding. Once coating on the glass surface, the hydrophilic part of the first layer faces to the surface, and the hydrophobic patch of the first layer turns outside; then the hydrophobic group of the second layer binds to the hydrophobic part of the first layer, and finally the hydrophilic part points toward outside. Similar theory has also been applied to explain the increase of WCA on the Teflon surface after coating with hydrophobin I SC3 [55].

3.3. Fluorescent image of printed protein

With the results of XPS and WCA, it can be concluded that modification with APTES or HFBI has definitely changed the surface property of glass slides. It is reported that both APTES modification and HFBI coating could help protein immobilization on solid surfaces [46,56]. Subsequently, we detected and compared the ability of modified glass surfaces to immobilize proteins.

Here, microcontact printing technology, introduced by Whitesides and co-workers in 1993 (see [48] paper references in [25]), was adopted to investigate protein immobilization on glass slides. Polydimethylsiloxane was originally used to form a patterned stamp, which transfers "ink" made up of alkanethiol molecules to gold surfaces. Later, this method was also applied to pattern protein and cells on various solid surfaces [51,57].

To verify protein immobilization on modified glass surfaces, microcontact printing was applied to detect immunoassays on the substrates, and printed proteins were observed with inverted fluorescence microscopy. The immunoassay result on APTES-modified surface was shown in Fig. 3, and that on HFBI-modified surface was shown in Fig. 4. The width of the strips is about 200 μm, which was consistent with that of the PDMS stamps. Since the patterns on the PDMS stamp could keep good mechanical stability and patterned proteins do not diffuse on the surface, the strips obtained with μCP showed high contrast and resolution with low noise signal. All sites left available could be blocked with bovine serum albumin after patterning the desired proteins [49], in order to prevent non-specific binding during later steps of the assay. Thus, after blocking with BSA, patterned chicken IgG was recognized by FITC-labeled anti-chicken IgG specifically. From the figure, it is clearly seen that only strip pattern is fluorescent, verifying the immobilization of chicken IgG on the modified glass slides.

Moreover, patterns obtained in Fig. 4 were more homogeneous than that obtained in Fig. 3. The difference in the efficiency of modification is one reason for this phenomenon. Under silanization treatment, chemical reaction occurred between APTES and the glass surface, which cannot react completely, and hydroxyl groups on the surface cannot be fully instead by the reactive amine group (supported by the data of XPS in Table 1). On the other hand, hydrophobin HFBI often forms a protein film on the glass surface, which covered the solid support greatly. Since only amine group or hydrophobin on the surface could help adsorb protein effectively, the protein strips in Fig. 3 were not as homogenous as that in Fig. 4.

The adsorption of protein on both the modified surfaces has different mechanism of immobilization. The interaction between

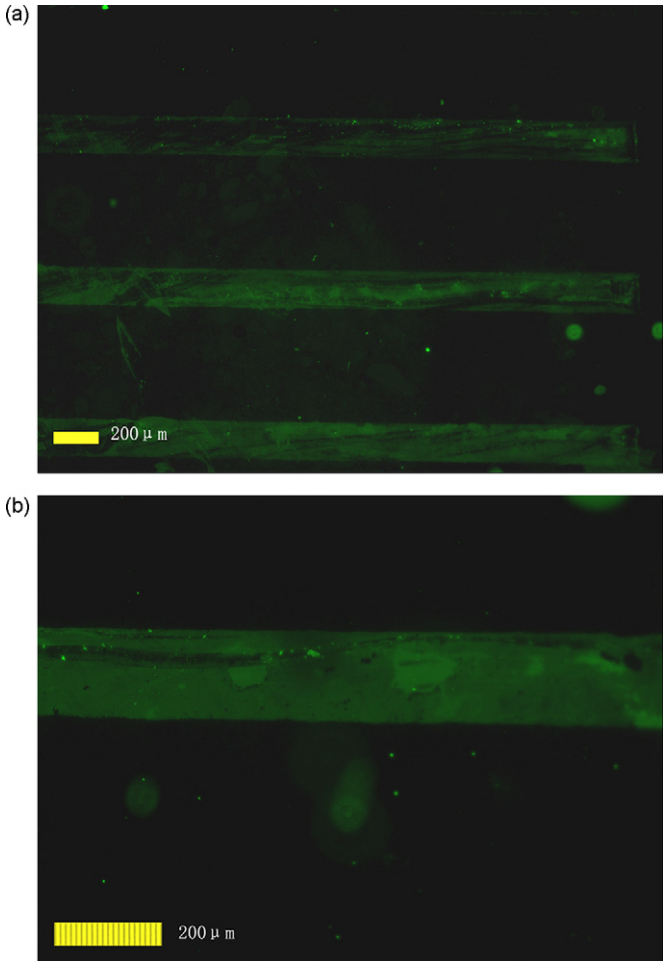

Fig. 3. Fluorescent images of patterned chicken IgG with blocked FITC-labeled anti-chicken IgG on the same APTES-modified glass slide. (The above are FITC images and are magnified 40 and 100 times, respectively.)

protein and APTES-modified surface is electrostatic interaction. Treatment with APTES causes reactive amine groups on the glass surface, which bring positive charge. The overall positive charge of amine surfaces allows attachment of proteins that carry negative charges, such as Glu and Asp. On the other hand, the principal of protein (chicken IgG) immobilization on the HFBI-coated glass substrate can be attributed to non-covalent interactions between proteins, including electrostatic interaction, hydrogen bonding and van der Waals forces.

3.4. Comparison of the two methods: APTES modification and HFBI coating

Originated from DNA microarray, amine treatment has been widely used in protein chips for its simple procedure and reliable property. With the results of immunoassays on APTES-modified surface, its effect on protein immobilization is clearly shown. However, amine treatment often results in protein denaturation. Besides, it requires the strict standard of cleanness before modification. With the results of XPS, WCA and immunoassay, we can concluded that HFBI coating can achieve the same result of protein immobilization on glass slides as APTES modifica-

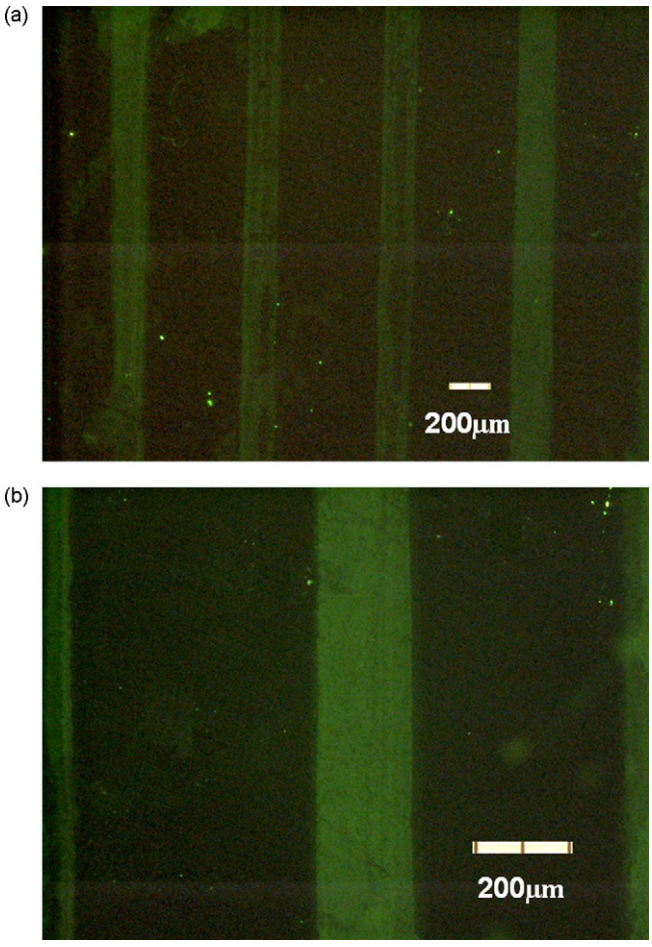

Fig. 4. Fluorescent images of patterned chicken IgG with blocked FITC-labeled anti-chicken IgG on the same HFBI-modified glass slide. (The above are FITC images and are magnified 40 and 100 times, respectively.)

tion did, or even better. Besides, HFBI coating also has some other advantages. The HFBI coating did not require the complex procedure and strict surface cleanness as the silanization needed. What is more, HFBI-coated surfaces can be used immediately or preserved for some days prior to use. Combined with the hydrophobins' amphiphilic property, they could help bind proteins on solid supports and can be applied in protein chip.

4. Conclusions

In summary, two methods for the modification of glass surfaces were analyzed, one is APTES modification, and the other is HFBI coating. The XPS and WCA results confirmed that both methods have changed surface properties of glass slides. The following immunoassays using μCP illustrated that both APTES modification and HFBI coating could immobilize proteins effectively. Besides, compared with the widely used amine treatment, we found that HFBI coating is a simple and generic way to immobilize protein on glass slides, and may improve the quality of immobilization. Hence, our research on the surface modification for protein immobilization might be useful for bioanalytical chemistry on solid supports, such as protein microarrays, microfluidics, and biosensors.

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