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ORIGINAL PAPER

Surface modification using a novel type I hydrophobin HGFI

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Abstract Surface wettability conversion with hydrophobins is important for its applications in biodevices. In this work, the application of a type I hydrophobin HGFI in surface wettability conversion on mica, glass, and poly (dimethylsiloxane) (PDMS) was investigated. X-ray photoelectron spectroscopy (XPS) and water-contact-angle (WCA) measurements indicated that HGFI modification could efficiently change the surface wettability. Data also showed that self-assembled HGFI had better stability than type II hydrophobin HFBI. Protein patterning and the following immunoassay illustrated that surface modification with HGFI should be a feasible strategy for biosensor device fabrication.

Keywords Hydrophobin · XPS · WCA · Self-assembly · Protein patterning

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Introduction

Fabrication of biocompatible surfaces with proper wettability is important for the applications in miniaturization of biosensors, clinical immunological assays, genome mapping as well as cell patterning [1, 2]. Recently, hydrophobins have received extensive attention as versatile materials for surface modification [2]. Hydrophobins are a family of small, cysteine-rich, and amphipathic fungal proteins [3], which have unique self-assembling behavior due to their remarkable structural characteristics [4]. Their molecular weight is about 7–15 kDa and their amino acid sequence has hydrophilic and hydrophobic regions. They can self-assemble into amphiphilic membranes at hydrophilic–hydrophobic interfaces to convert the surface from hydrophilic to hydrophobic and vice versa [4]. There are two types of hydrophobins: type I and type II, which are classified according to their self-assembly patterns and solubility characteristics [5]. The type I hydrophobins, such as SC3, can form random patterns of nanoscopic rodlets and bundles when dried on the hydrophilic surfaces [6, 7]. The type II hydrophobins, such as HFBI and HFBII, can form highly ordered monolayer films and crystalline fibrils when assembled at an interface [8, 9]. Besides, aggregates of the type I hydrophobins are very insoluble, such as SC3, which can only be solved in strong acids [10]; while aggregates of the type II hydrophobins are easier to be solved and can be solved in ethanol or sodium dodecyl sulfate [11]. Researches on the applications of hydrophobins in biocompatible surface modification will be of great interest in the field of biosensor fabrication and tissue engineering.

As far as the surface modification is concerned, mica, glass, and PDMS are three commonly used surfaces that stand for extremely hydrophilic surface, hydrophilic surface

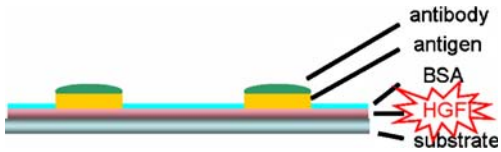
and hydrophobic surface respectively. Mica is of atomic-scale flatness [12, 13]. Freshly cleaved mica is a promising substrate for patterning applications. Glass is a common material for cell culture and protein fixation [14]. Modified glass with improved property, for example, changed wettability, is suitable for cell and protein patterning. PDMS is a cheap, transparent, flexible material that has been used extensively in medical implants and biomedical devices because of its biocompatibility [15, 16], low toxicity [17, 18] and high oxidative and thermal stability [19, 20]. Unlike mica and glass, the PDMS surface is very hydrophobic. The hydrophobicity of the PDMS surface will weaken the immobilization of biomaterials. Treatment must be applied on the PDMS surface to improve its wettability, such as oxidation of the surface by ultraviolet [21, 22], plasma treatment [23, 24], CO_2 pulsed laser [25], and grafting polymer on PDMS [26].

In this study, we examined the surface modification properties of a type I hydrophobin HGFI. HGFI is distilled from the edible mushroom *Grifola frondosa* [27], which is presented as two separate peaks obtained after HPLC of mycelial extracts: components a and b. Mica, glass, and PDMS were used as the extremely hydrophilic substrate, the hydrophilic substrate, and the hydrophobic substrate to investigate the self-assembly of HGFI on these surfaces, respectively. XPS and WCA measurements were used to monitor the surface modification process by HGFI. We also compared the stability of the self-assembled film (SAM) of HGFI with that of a type II hydrophobin HFBI to show the superiority of HGFI in surface modification. Furthermore, protein immobilization and immunoassay experiment on three HGFI-modified surfaces were employed to illustrate the application of the HGFI-modified surfaces (Scheme 1).

Experimental

Materials

A PDMS elastomer kit (Sylgard 184, Dow Corning), curing agent, bovine serum albumin (BSA), chicken immunoglobulin (IgG) and fluorescein isothiocyanate (FITC)-labeled anti-chicken IgG from rabbits were purchased from Sigma. Chicken IgG was used as an antigen and FITC-labeled anti-chicken IgG was used as an antibody. The concentrations of chicken IgG and FITC-labeled anti-chicken IgG were 200 μg/mL. The concentration of BSA was 10 mg/mL. All proteins were dissolved in phosphate-buffered saline (PBS) solution at pH 7.4. HGFI was dissolved in Milli-Q water to a concentration of 100 μg/mL. The pH of the water used for washing was 7.4. The details of the preparation of HGFI and the procedure for high-performance liquid chromatography analysis to separate the two components of HGFI were the same as we reported [27].



Scheme 1 Surface modification with HGFI for protein immobilization and immunoassay

HGFI self-assembly on multiple substrates

HGFI self-assembly on the mica surface: Mica was cleaved with a stainless steel razor blade. The mica slices were incubated in a solution of HGFI for 20 min at 20 °C. Then, the substrates were dried with a stream of nitrogen. The dried surface was gently rinsed with water to remove unbounded HGFI. Finally, the surface was dried once more with a stream of nitrogen gas.

HGFI self-assembly on the glass surface: The glass slices were rinsed with water and then ethanol for several times. Then, the substrates were dried with a stream of nitrogen gas. The HGFI self-assembly procedure was the same as that on the mica surface.

HGFI self-assembly on the PDMS surface: Silicon elastomer and the curing agent (10:1 *w*/*w*) were poured onto the glass substrate after being mixed sufficiently. The substrate was cured overnight at 60 °C. Then, the PDMS was obtained after peeling off the glass substrate. Before modification, the PDMS was rinsed several times with water and ultrasonicated for 10 min. Then, the PDMS was rinsed with 75% ethanol solution for several times, followed by drying with a stream of nitrogen. The HGFI self-assembly procedure was the same as that on the mica surface.

XPS measurements

The XPS measurements were carried out by using an XPS apparatus (PHI-5300, Phi USA) employing a monochromatic Mg Kα radiation source (1,253.6 eV). The survey scan range was 0–1,100 eV. The electron takeoff angle was fixed at 45°. The energy resolution of the analyzer was 0.8 eV. The sensitivity was 80–1,600 kcps. The peaks in the elemental core level spectra were fitted by using UNIX on an Apollo Domain series 3500.

WCA measurements

The static WCA measurements were carried out with 5-μL water droplets at ambient temperature by using an optical

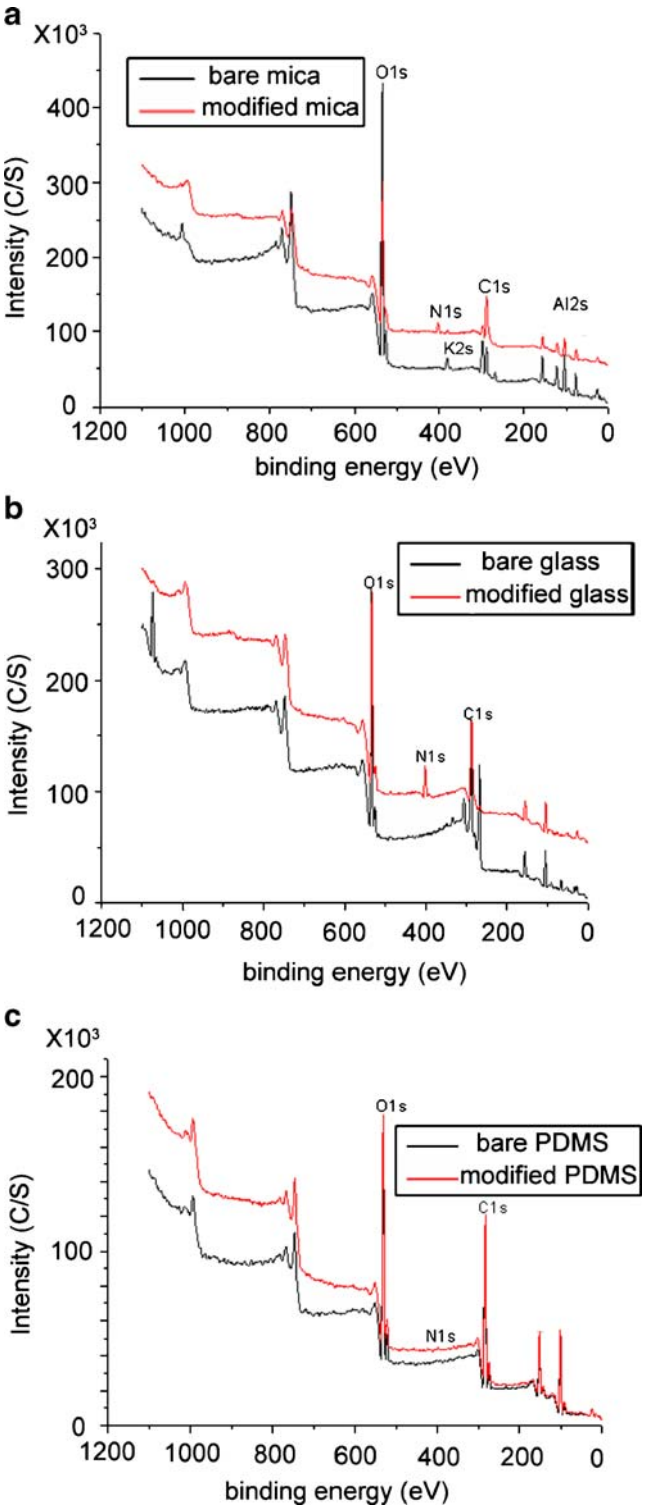


Fig. 1 XPS spectra of **a** a mica surface, **b** a glass surface and **c** a PDMS surface before and after HGFI modification

contact angle meter (Dataphysics Inc, OCA20). The WCA were averaged from three individual measurements at different locations. All the measurements were within ±3° off the average.

Stability of HGFI self-assembly

Self-assembly of HGFIa, HGFIb, and HFBI were achieved on silicon glasses HR3-239 (Hampton research, United States). The concentration of HGFIa, HGFIb, or HFBI was 100 μg/mL. The self-aseembly of HGFIa, HGFIb, and HFBI on the siliconized glasses was prepared in the same way as HGFI on the mica surfaces. The stability was tested by monitoring the WCA changes after rinsing. In brief, these coated surfaces were rinsed three times with 2% hot (80 °C) SDS solution and 60 vol.% ethanol solution, respectively. The WCA measurements were carried out with KSV Cam200 goniometer (KSV Instruments, Finland) and results were calculated with the software provided by manufacturer. At least three measurements were analyzed on different locations on the sample surface. All measurements were within ±3.0° off the averages.

Protein immobilization and immunoassay on HGFI-modified mica and glass surface

The PDMS stamp was prepared by curing a mixture of liquid PDMS and cross-linker (10:1 *w*/*w*) on the silicon master at 60 °C overnight. Then, the silicon master was peeled off and the PDMS stamp was obtained. The silicon master had depressed parallel strips of 100 μm width. Consequently, the resulting PDMS stamp had extruded parallel strips by replicating the surface feature of the silicon master.

Mica and glass surfaces coated with HGFI were prepared in the same way as described above ("HGFI self-assembly on multiple substrates"). Chicken IgG was immobilized by μCP [28]. In brief, the PDMS stamp was cleaned several times with distilled water and then with 75 vol.% ethanol. Then, the stamp surface was inked in the chicken IgG solution for 2 min. Excess solution on the stamp was removed with a piece of lens paper. Then, the stamp was gently pressed on the HGFI-coated mica or glass substrate. The chicken IgG molecules were transferred from the stamp bottom onto the substrate surface. The patterned surface was blocked with BSA solution for 20 min, followed by rinsing three times with water and

Table 1 XPS determination of the relative atomic composition

	N (%)	O (%)	Al (%)
Bare mica	0.04	56.98	12.86
HGFI-modified mica	3.90	40.00	7.96
Bare glass	0.20	54.91	–
HGFI-modified glass	7.16	34.87	–
Bare PDMS	0.00	27.61	–
HGFI-modified PDMS	0.65	28.01	–

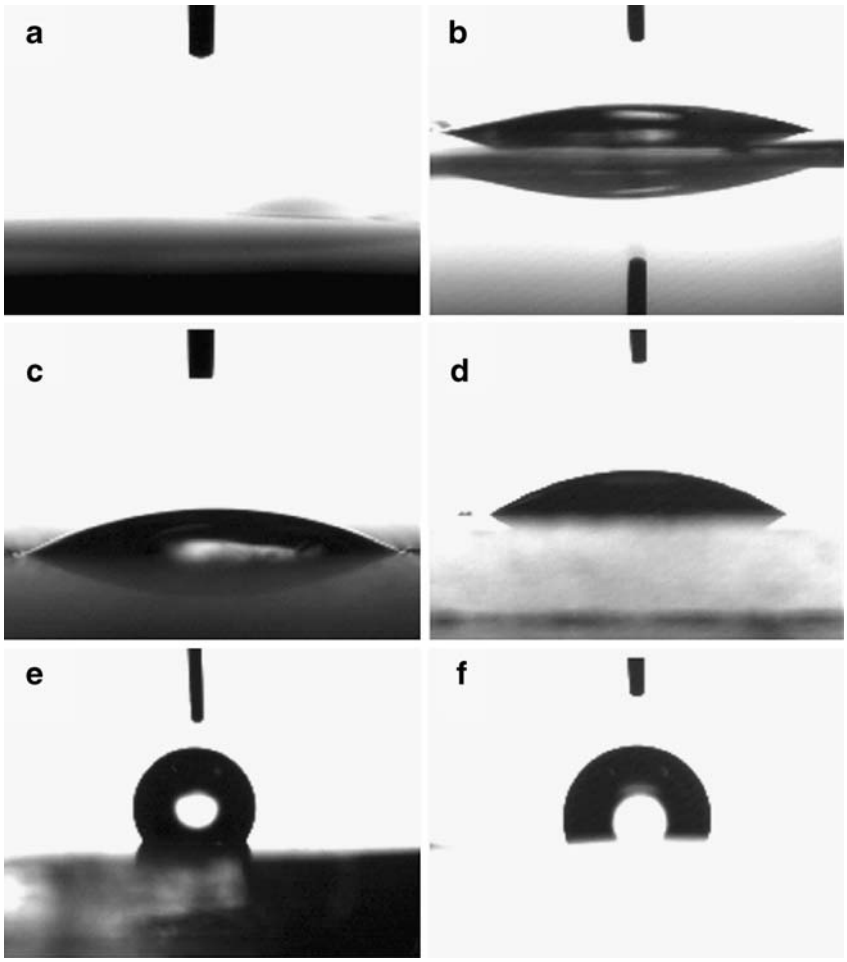


Fig. 2 WCA measurement of multiple surfaces modified by HGFI. **a** A bare mica surface. **b** A HGFI-modified mica surface. **c** A bare glass surface. **d** A HGFI-modified glass surface. **e** A PDMS surface. **f** A HGFI-modified PDMS surface

drying with a stream of nitrogen gas. FITC-labeled anti-chicken IgG was coated on the substrate for 2 min and then the substrate was rinsed with water to remove excess antibody. The immunoassay result was observed under an inverted fluorescence microscopy (Nikon TE2000-U, CCD-RtKe, Japan).

Protein immobilization and immunoassay on HGFI-modified PDMS surface

PDMS surfaces coated with HGFI were prepared in the same way as described above ("HGFI self-assembly on multiple substrates"). Since PDMS was not rigid enough for μCP procedure, we used an alternative method to pattern chicken IgG. The TEM copper grids were pressed onto the PDMS surface using a piece of glass. The PDMS surface was coated with HGFI solution using the same method as described above. Then, the chicken IgG solution was coated on the substrate for 2 min, followed by blowing off excess solution with a stream of nitrogen gas. After the copper grids were peeled off, the protein pattern on PDMS was obtained. This surface was blocked with BSA solution for 20 min. Then, the substrate was rinsed with water for three times and dried with a stream of nitrogen gas. FITC-labeled anti-chicken IgG was coated on the substrate for 2 min and then the substrate was rinsed with water to remove excess antibody. The immunoassay result was observed under an inverted fluorescence microscopy (Nikon TE2000-U, CCD-RtKe, Japan).

Results and discussions

Characterization of HGFI modification on multiple surfaces by XPS

Surface modification with HGFI was investigated via monitoring the changes of the surface chemical composition by XPS measurement (Fig. 1 and Table 1). A higher-intensity O 1*s* peak associated with lower-intensity of Al 2s peak represented a characteristic XPS spectrum of the mica surface. After HGFI modification, the intensity of N 1*s* signal increased dramatically (from 0.04% to 3.90%) at 400 eV. N belongs to the HGFI and there is nearly no N element in the mica. The increase of N 1*s* signal indicated that HGFI was immobilized on the mica surface. This conclusion could be supported by the fact that the intensity

Table 2 WCA of multiple surfaces modified by HGFI

	Bare surface (°)	Modified by HGFI (°)
Mica	0.3	17.9
Glass	25.7	30.6
PDMS	123.9	104.5

All measurements were within ±3.0° off the averages

of Al 2s signal was weakened after HGFI modification (from 12.86% to 7.96%). Al existed in mica but not in HGFI. The decreased Al 2s signal confirmed that HGFI had covered the mica surface to some extent.

Similar XPS results were obtained on bare glass surface and HGFI-modified glass surface. The intensity of N 1*s* signal increased dramatically (from 0.2% to 7.16%) at 400 eV after HGFI modification, which indicated that HGFI could be immobilized on the glass surface by this method.

XPS results on PDMS surface showed a slight increase of N 1*s* signal after HGFI modification. The intensity of N 1*s* increased from 0% to 0.65%. PDMS did not contain N element. The appearance of N 1*s* indicated that HGFI could be immobilized on the PDMS surface; however, it did not cover the surface to a great extent (detailed elemental scans of N 1*s*, Si 2*p* and Al 2*p* on mica, glass and PDMS surfaces can be found in the Supplementary Data).

WCA measurement

Surface wettability shift is an important function of surface modification with hydrophobins. The surface wettability of multiple surfaces modified by HGFI was investigated by WCA measurement (Fig. 2 and Table 2).

The WCA of the bare mica surface was about 0.3°, which indicated that the mica surface was extremely hydrophilic. After HGFI modification, the WCA of the mica surface increased to about 17.9°. This result indicated that the hydrophilicity of mica surface was decreased by HGFI modification. The mica surface is of atomic-scale flatness. HGFI immobilization may increase the surface roughness. In Wenzel's equation [29],

$$\cos\theta' = r\cos\theta$$

where θ' is the apparent WCA on a rough surface and θ is the intrinsic on a flat surface, the surface roughness (r) can enhance both the hydrophilicity on a hydrophobic surface and the hydrophobicity on a hydrophobic surface. Herein, the roughness will decrease the WCA of mica surface. However, in the study, the WCA was increased after surface modification with HGFI. The changes of the WCA on the hydrophilic mica surface were supposed to be caused by the amphiphilic properties of hydrophobins. When self-assembling on the hydrophilic surface, the hydrophilic regions of HGFI were attached to the hydrophilic surface and the hydrophobic regions of hydrophobins were exposed to the outside, which increased the surface hydrophobicity.

A similar result was obtained on the glass surface. The hydrophilic nature of the bare glass surface was indicated by showing a WCA of about 25.7°. After HGFI modification, the WCA increased to about 30.6°. This result indicated that HGFI was immobilized on the glass surface. The changes of the WCA were also expected to be caused by the amphiphilic properties of hydrophobins. However, the WCA changes on the glass surface were not as large as that on the mica surface.

PDMS surface was hydrophobic with a WCA of 123.9°. After HGFI modification, its WCA decreased to 104.5°. The change of WCA was related to the amphiphilic property of HGFI assembly. When hydrophobins self-assembled on the hydrophobic surface, it was supposed that the hydrophobic regions of HGFI were attached to the hydrophobic surface and the hydrophilic regions of hydrophobins were exposed to the outside [2, 14, 21]. This process decreased the WCA of the hydrophobic surface. The resulting surface was of moderate wettability and herein could be used for proteins immobilization.

Stability of HGFI self-assembly

The stability of HGFI self-assembly was investigated on a silicon glass surface by monitoring the WCA changes after rinsing by several solutions (Table 3). HGFI composed of two components: components a and b (Fig. 3). Surface modification using HGFI was presented as the compound effect of both components a and b, which would self-assemble on the interfaces due to their amphiphilic properties. There is no necessary need to separate them before use. However, we found the two components might have different stabilities. In order to probe their detailed properties, the stabilities of these two components were investigated, respectively.

Table 3 Stability analysis of HGFI and HFBI coating by WCA measurement

	Component a (°)	Component b (°)	HFBI (°)
Before coating	86.6	86.6	86.6
After coating	51.9	44.3	30.1
Rinsed with 60% ethanol	80.3	46.3	79.8
Rinsed with 2% hot SDS	76.1	49.3	83.4

All measurements were within ±3.0° off the averages

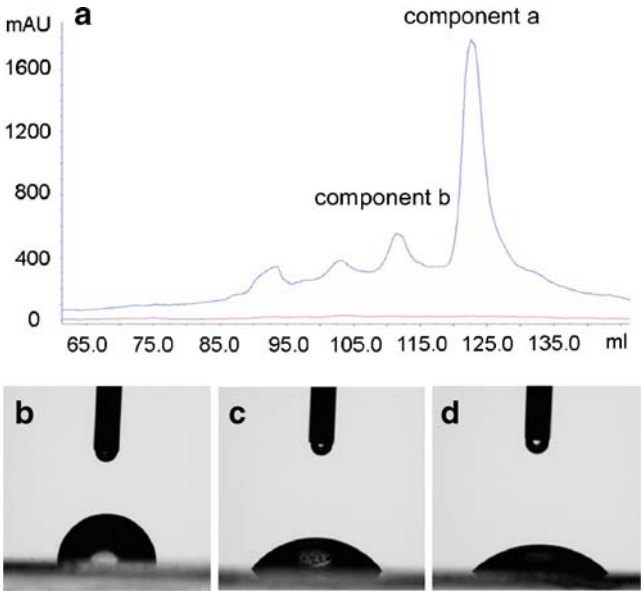


Fig. 3 **a** High-performance liquid chromatography of HGFI. **b** WCA of bare silicon glass. **c** WCA of component a modified silicon glass. **d** WCA of component b modified silicon glass

The silicon glass surface showed a WCA of 86.6°. Surface modification with component a, component b, and HFBI efficiently changed the WCA to 51.9°, 44.3°, and 30.1°, respectively. Self-assembly of hydrophobins would form an amphiphilic film on the surface of silicon glass, which was supposed to cause the changes of the WCAs. After rinsing with 60% ethanol, the WCA of modified silicon glasses increased. The restoration of WCA was due to the remove of hydrophobins from the silicon glass. The WCAs of component a and HFBI modified surface were 80.3° and 79.8°, respectively, which were almost the same as the bare silicon glass. However, the WCA of component b modified surface was 46.3°, which increased just 2°. The result suggested that component b had a much better stability than HFBI and component a after rinsing with 60% ethanol. A similar experiment was performed with hot SDS solution. After rinsing with SDS, the WCA of component a and HFBI modified surface increased dramatically; while the WCA of component b modified surface just increased 5°. This result indicated that component b had a much better stability than HFBI and component a after rinsing with hot SDS solution.

Protein immobilization and immunoassay.

HGFI modification could improve the hydrophilicity on multiple surfaces. It has been reported that the hydrophilicity of the surfaces will facilitate adsorption of proteins. For example, it has been shown that wettability conversion on PDMS surface will facilitate protein adsorption [30]. The feasibility of using these modified surfaces for protein immobilization was investigated by immunoassay.

Chicken IgG was transferred onto the HGFI-modified mica and the HGFI-modified glass surfaces via a PDMS stamp using μCP technique [28]. After blocking the surfaces with BSA, we incubated the substrate in the corresponding FITC-labeled antibody solution. Because of their specificity in immunoassay, fluorescent antibodies only bound to the corresponding antigens. Consequently, the antigen patterns could be observed under a fluorescence microscope by detecting the antibodies.

In Fig. 4a and b, the fluorescent protein patterns on mica and glass surfaces showed high contrast and resolution with a clean background. The characteristic dimensions of the fluorescent stripes were consistent with that of the PDMS

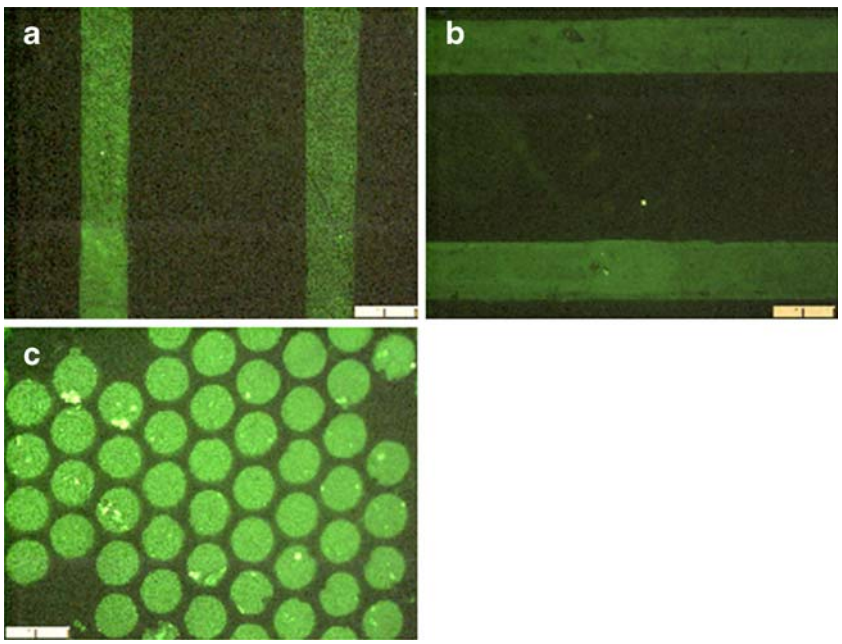


Fig. 4 Fluorescent images of patterned chicken IgG with blocked FITC-labeled anti-chicken IgG on a HFBI patterned **a** mica surface, **b** glass surface, and **c** PDMS surface. The *scale bar* is 200 μm

stamps. The result indicated that HGFI-modified mica and glass surfaces were suitable for protein immobilization.

Due to the elastic property of PDMS, we used an alternative method for protein patterning. TEM copper grids were pressed on the HGFI-modified PDMS surface. Then the surface was incubated with antigen solution. The antigen could only be immobilized on the place without grids. After the copper grids were peeled off, the surface was blocked with BSA and then incubated with the corresponding fluorescent antibody solution. Due to their specificity in immunoassay, the antibodies only bound to their antigens. Consequently, the fluorescent image described the pattern of antigens well. In Fig. 4c, the dimension of fluorescent pattern consisted with that of copper grids. The result indicated that HGFI-modified PDMS surface was suitable for protein immobilization. On the hydrophobic PDMS surface, the hydrophilic part of HGFI turned to the outside. Consequently, proteins could interact with the surface through polar interactions, including electrostatic interaction, hydrogen bonding, and van der Waals forces.

Compared with previous work about surface modification for protein immobilization [22, 23], surface modification with hydrophobins showed many advantages. It does not require the complex procedure and strict surface cleanness as the silanization. The resulting surfaces can be used immediately. The surfaces can be preserved for some days [14], etc. Compared with type II hydrophobin HFBI, type I hydrophobin HGFI showed its advantage: the stability of HGFI self-assembly was much stronger than HFBI. Although HGFI showed lower efficiency in wettability improvement on the PDMS surface than HFBI, the HGFI-modified PDMS surface was suitable for protein immobilization. Surface modification with HGFI might be an alternative strategy for protein immobilization. The application of this modification method could be adapted to a number of related research topics, such as biodevice fabrication, microfluidic system and clinic immunoassay.

Conclusions

Surface modification with HGFI for protein immobilization was investigated. HGFI modification was applied on multiple surfaces, ranging from the hydrophobic PDMS surface to the hydrophilic mica surface. Data from XPS and WCA measurements confirmed the feasibility of using HGFI to convert the wettability of the surfaces. HGFI self-assembly showed admirable stability against rinsing by several solutions. The following immunoassay experiment illustrated that the HGFI-modified surfaces could be used for protein immobilization. These advantages enabled HGFI to be a useful material for surface modification with potential applications in biodevice fabrication and immunoassay.

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