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Phage M13KO7 detection with biosensor based on imaging ellipsometry and AFM microscopic confirmation

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ABSTRACT

A rapid detection and identification of pathogens is important for minimizing transfer and spread of disease. A label-free and multiplex biosensor based on imaging ellipsometry (BIE) had been developed for the detection of phage M13KO7. The surface of silicon wafer is modified with aldehyde, and proteins can be patterned homogeneously and simultaneously on the surface of silicon wafer in an array format by a microfluidic system. Avidin is immobilized on the surface for biotin-anti-M13 immobilization by means of interaction between avidin and biotin, which will serve as ligand against phage M13KO7. Phages M13KO7 are specifically captured by the ligand when phage M13KO7 solution passes over the surface, resulting in a significant increase of mass surface concentration of the anti-M13 binding phage M13KO7 layer, which could be detected by imaging ellipsometry with a sensitivity of 10^9 pfu/ml. Moreover, atomic force microscopy is also used to confirm the fact that phage M13KO7 has been directly captured by ligands on the surface. It indicates that BIE is competent for direct detection of phage M13KO7 and has potential in the field of virus detection.

1. Introduction

Recently, a great deal of attention has been paid to early recognition of epidemic diseases, environmental contamination or viral bioterrorist attack. Rapid detection and identification of pathogens are critically important not only to minimize transfer and spread of disease but also to devise and evaluate effective treatment strategies. The standard laboratory diagnostic test for viruses is based on their culture (Henderson et al., 1999), which is very sensitive but cumbersome and time-consuming. Labeling methods such as fluorescent technique, electrochemiluminescence and immunosorbent assays occasionally results in false positives or a high percentage of false negatives (Long et al., 2004; Wang and Jin, 2004; Wu et al., 2004). Label-free methods such as quartz crystal microbalance (QCM), magnetic bead cell sorting, atomic force microscope (AFM), polymerase chain reaction (PCR) and surface plasmon resonance (SPR) are advantageous in that the detection protocols are simplified through removing the need to label secondary antibodies (Bisoffi et al., 2008; Nettikadan et al., 2003; Oh et al., 2003; Poon et al., 2004; Porter and Pickup, 1998; Uttenthaler et al., 2001). However, some of them need expensive equipment, associated specialist skills and complicated sample preparation process. For example, AFM and PCR needs relatively expensive equipment and associated specialist skills to perform the analysis. SPR is confined to gold-thiols self-assembly in the analysis. In order to avoid the problems, there is an urgent need for rapid and sensitive testing, multiplex and simple operation for virus detection.

Developed in 1995 (Jin et al., 1996, 1995), the biosensor based on imaging ellipsometry (BIE) employs a protein analysis technique that combined high spatial resolution imaging ellipsometry with multi-protein microarray. BIE has been mainly applied to biomedical fields, such as (1) biomolecule interaction (Qi et al., 2006a; Wang and Jin, 2003b). (2) Hormone detection (Zhao et al., 1998). (3) Cell factor and its receptor interaction (Wang and Jin, 2002). (4) Cancer markers test (Zhang et al., 2005). (5) Clinical diagnosis for hepatitis B (Jin et al., 2004). (6) Quantitative protein competitive adsorption (Ying et al., 2004). (7) Kinetic detection for multi-protein interac-

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tion process (Jin et al., 2003). (8) Bacterium detection (Bae et al., 2005) and others.

In this study, oriented immobilization of ligand was realized by means of interaction between avidin and biotin. An ultrasonic method was adopted to disperse phage sample to corresponding areas of protein microarray, so that it would be homogeneous. Different phage M13KO7 samples, such as natural and purified samples and different batches of samples with varied concentrations, were definitely distinguished by BIE. More importantly, the results of BIE were confirmed at a microcosmic level by AFM, which could realize a microcosmic observation of the interaction between proteins and viruses.

2. Materials and methods

2.1. Biosensor substrate, chemicals and phage sample

Silicon wafers were purchased from Luoyang Monocrystalline Silicon Factory; 3-aminopropyltriethoxy-silane (APTES, 99%, v/v) from ACROS, H_2O_2 (30%), H_2SO_4 (98%) and absolute ethanol were from Beijing Bei Hua Fine Chemicals Co., Ltd.; Glutaraldehyde (50% aqueous solution photographic), avidin, human IgG, bovine serum albumin (BSA) and tween 20 were from SIGMA. All chemicals were of analytical grade. Antibody (GP3) against phage M13KO7 and GP3 labeled with biotin (Bio-GP3) were from Exalpha Biologicals, which could specifically identify minor coat protein pIII on the head of phage M13KO7 (Van Wezenbeek and Schoenmakers, 1979). Natural and purified phage M13KO7 (5×10^{11} pfu/ml) samples, severe acute respiratory syndrome (SARS) virus were provided by the Institute of Biophysics, Chinese Academy of Sciences (Kuznetsov et al., 2001). Phosphate-buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and PBST (PBS with 1% tween 20) were prepared in deionized water (Millipore, Resistivity 18.3 MΩ cm). All samples were diluted with PBST. Ultrasonic machine was produced by Jiangsu Kunshan Ultrasonic Instruments Co., Ltd., China. AFM experiments were performed by Institute of Chemistry, CAS using AFM purchased from Digital Instrument USA.

2.2. Detection procedure of BIE

Firstly, silicon wafers were chosen as the substrate, and then modified with chemical reagents to form chemical groups on its surface in order to covalently immobilize proteins as ligands. Secondly, the modified wafer was put into a microfluidic system so that its surface was patterned to form regular small areas. Thirdly, antibody against phage M13KO7 was immobilized to form a sensing surface; phage samples were then added to the sensing surface. If the samples contained phage M13KO7, the phages would be specifically captured by the sensing surface. Finally, the wafer was taken out from the microfluidic system and submitted to imaging ellipsometry for analyzing.

2.3. Surface modification of silicon wafer

The silicon wafers were cut into rectangles 20 mm × 10 mm. The surface of wafers was washed in solution (H_2O_2:H_2SO_4 = 1:3, v/v) for 30 min. The solution not only removed contaminants on the surface of silicon wafers but also improved the number of silanol groups. After being rinsed in deionized water and ethanol, the wafers were incubated in a mixture of APTES and absolute ethanol (APTES:absolute ethanol = 1:10, v/v) for 2 h, and then the wafers were rinsed again in absolute ethanol three times. APTES would react covalently with silanol groups on the surface to produce –O–Si(OH)$_2$–(CH$_2$)$_3$–NH$_2$, which would then be immobilized and formed a layer of densely packed amino groups on the silicon dioxide layer. After rinsed in ethanol and PBS, the washed wafers were incubated in a mixture of Glutaraldehyde and PBS (Glutaraldehyde:PBS = 1:15 v/v) for 1 h, and in this period OHC–(CH$_2$)$_3$–CHO of Glutaraldehyde could react with –NH$_2$ of –O–Si(OH)$_2$–(CH$_2$)$_3$–NH$_2$ immobilized on the surface to produce –(CH$_2$)$_3$N=CH(CH$_2$)$_3$–CHO (Wang and Jin, 2004). After rinsed with PBS, the wafers were kept in PBS.

2.4. Patterning surface with microfluidic system for parallel immunoassays

Proteins can be patterned homogeneously and simultaneously on the substrate in an array format by the microfluidic system. After preparation, the protein array can be packed in the microfluidic system, which is full of buffer so that the protein will not be exposed to denaturing conditions. With simple microfluidic channel junction, the protein microfluidic array can be used in serial or parallel formats to analyze single or multiple samples simultaneously. Please refer to relevant literature (Wang et al., 2006) for technical details.

2.5. Immobilization of ligand

With the above-mentioned method of patterned surface, bio-GP3 was immobilized as ligand and formed different areas on the substrate by the avidin–biotin model, which was a routine method of ELISA, RT-PCR, biosensor, etc. for binding antibody (Gao et al., 2002; Hu et al., 2002; Zacco et al., 2006). The avidin (0.5 mg/ml, 10 μl, passing the surface at 2 μl/min) could be covalently immobilized on the modified substrate due to the reaction of Schiff base with –CHO. The areas were rinsed with deionized water, and each area was then blocked with 10 mg/ml BSA for 40 min. When the areas were blocked, 0.1 mg/ml bio-GP3 was added (10 μl, 2 μl/min). Because bio-GP3 on these areas could specifically identify minor coat protein pIII on the head of phage M13KO7, some identical immune-probes of phage M13KO7 were formed, which were capable of detecting separate samples simultaneously.

2.6. Phage M13KO7 detection with BIE

After the immune-probes were formed, some areas were chosen to detect phage solution and others were used as control. Phage M13KO7 culture medium and negative samples were added into each sensing immune-probe (50 μl, 1 μl/min), respectively. Immune-probe could capture phage M13KO7 in the solution and form a complex, which caused the surface concentration of the layer to increase, and thus the value in gray-scale would increase accordingly, indicating the fact that the solution did contain phage M13KO7. After being taken from the microfluidic system, the wafer was first rinsed with deionized water and then blown with nitrogen, and was finally analyzed by the imaging ellipsometry.

Imaging ellipsometry is a technique for displaying characteristics of ultra-thin films and surfaces (Arwin et al., 1993; Jin et al., 1996, 1995; Stenberg and Nygren, 1983; Wang and Jin, 2003a). The incident wave of polarized light irradiates the sample as probe beam and is thereby modified, which makes the reflective or transmission beam carry sample information, such as protein layer thickness. When imaging ellipsometry is used to detect layer thickness, the reflection intensity is represented by gray-scale. Variation of layer thickness leads to change of gray-scale value. Suppose the refractive index is invariant, the gray-scale value is directly proportional to the square of the protein layer thickness in the range of 0–30 nm layer thickness, i.e. $I = kd^2$, where I is the light intensity and d is the layer thickness (Arwin et al., 1993). Under the same conditions for protein and ellipsometry, k is constant and can be determined from a protein layer with known gray-scale and known thickness (Arwin et al., 1993). On the other hand, there is a relation between surface concentration and film thickness: surface concentration

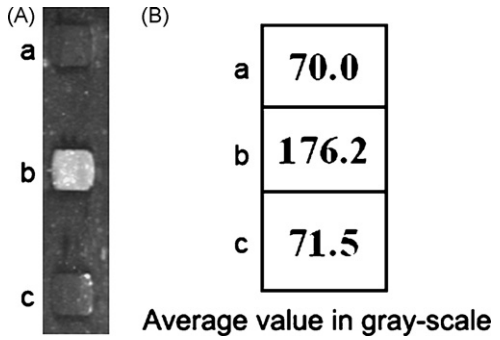

Fig. 1. Detection of different batches of phage M13KO7 samples. (A) The image in gray-scale; (B) Average value in gray-scale of the image "A". Antibody GP3 was immobilized on areas "a, b, c", area "a" was GP3 layer as a control, area "b" and "c" was used to detect the first and second batches of phage, respectively.

($\mu g/cm^2$) $\approx K \times d$, where $K = 0.12$ (Stenberg and Nygren, 1983; Ying et al., 2004). And thus the gray-scale value reflects directly the layer thickness or the surface concentration. The higher the gray-scale value, the thicker the layer and the higher the surface concentration.

2.7. Confirmation with AFM

The areas of antibody and phage were imaged with AFM in tapping mode, and different sizes and frequencies were used for optimal results. Firstly, the phage sample was added on the mica surface and scanned with AFM under different sizes (1.2 and 5 μm) and different frequencies (1.209 and 0.8846 Hz), which could offer original configuration of phage M13KO7 as the control image. The antibody GP3 on silicon was scanned under 500 nm and 1.606 Hz as negative control. The phage detection area on the silicon wafer was scanned under 5 μm and 1.327 Hz.

3. Results

3.1. Constitution of the sensing microarray

Biotin and avidin can specifically interact with their affinity (about 10^{15} M^{-1}). Every avidin molecule has four interaction locations to be combined with biotin. When biotin is combined with high molecular weight, the biotin molecule can still be identified by the avidin. Using the method, oriented immobilization of bio-GP3 can be realized. We used the surface of silicon as a sensing surface to catch phage M13KO7, as described in references (Bisoffi et al., 2008; Qi et al., 2006b).

3.2. Detection of phage M13KO7

In order to test different bioactivity, different batches of phage samples were prepared for detection, and the result was shown in Fig. 1. The first batch of phage samples did cause variation of value in gray-scale (about 151%), indicating that the samples contained phage M13KO7 with bioactivity (see "area b" in Fig. 1). The second batch of phage samples did not cause obvious variation of value in gray-scale (about 2%), which showed that the second batch did not contain phage M13KO7 or the phage had lost its activity (see "area c" in Fig. 1). Purified phage M13KO7 was detected three times and the results were shown in Fig. 2. Different concentrations of avidin were used (0.5, 1, 1.25 mg/ml), resulting in different value in gray-scale.

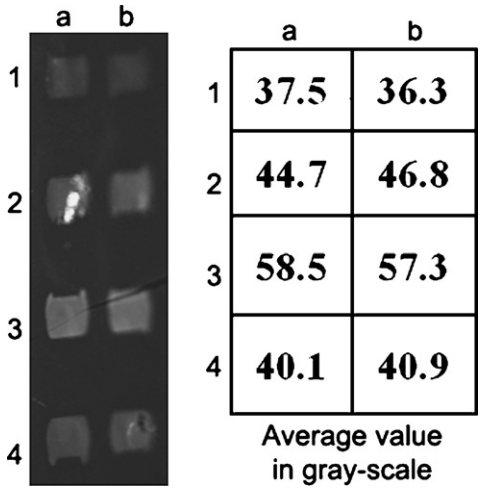

Fig. 3. Detection of phage M13KO7 (the sample was dispersed by ultrasonic). Avidin was firstly immobilized on all areas, and then bio-GP3 was immobilized on areas "a2, a3, a4, b2, b3, b4". In the detection, areas "a2, b2" were added with PBST as control; areas "a3, b3" and "a4, b4" were used to detect phage M13KO7 and negative sample (SARS), respectively.

Small white spots on the area of phage detection in Fig. 1 might be aggregates of some filamentous phages, which could be dispersed by ultrasonic of 90 Hz. Corresponding areas for phage detection were homogeneous, as shown in Fig. 3. Moreover, SARS virus (Duan et al., 2006, 2005) as negative sample was also detected with bio-GP3 as ligand (areas a4 and b4 in Fig. 3). Compared with the bio-GP3 layer, the surface concentration of the layer for detecting SARS did not have significant increase. Different concentrations of phage M13KO7 under detection may result in different increases in surface concentrations, as listed in Table 1. The sensitivity was 10^9 pfu/ml and the resolution was 0.7×10^9 pfu/ml.

3.3. Confirmation of the detection of phage M13KO7 by AFM

The major objective of this section was to utilize the micro-cosmic AFM image to confirm BIE's high medical and bio-warfare accuracy and its importance in detecting viruses. Fig. 4 shows the original and overall configuration of phage M13KO7 on the

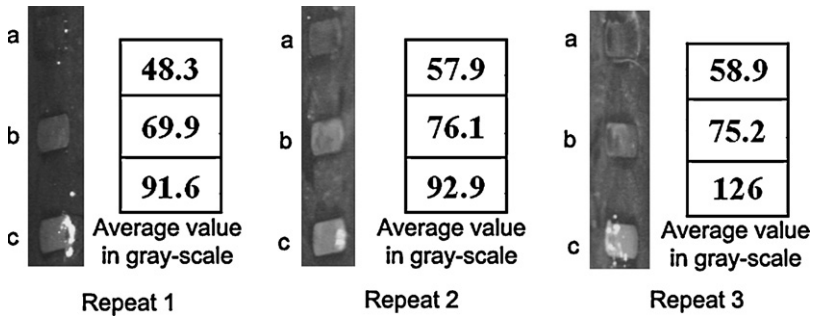

Fig. 2. Detection of purified phage M13KO7. Avidin was firstly immobilized on areas "a, b, c", and then areas "b" and "c" were used to immobilize GP3, and area "c" was used to detect purified phage M13KO7.

Table 1
Detection of phage M13KO7 with different concentrations by BIE.

Concentrations of phage M13KO7 sample ($\times 10^{11}$ pfu/ml)	0	0.0125	0.126	0.732	2.5
Surface concentration (μg/cm^2) acquired by BIE detection	0	0.18	0.46	1.02	2.1

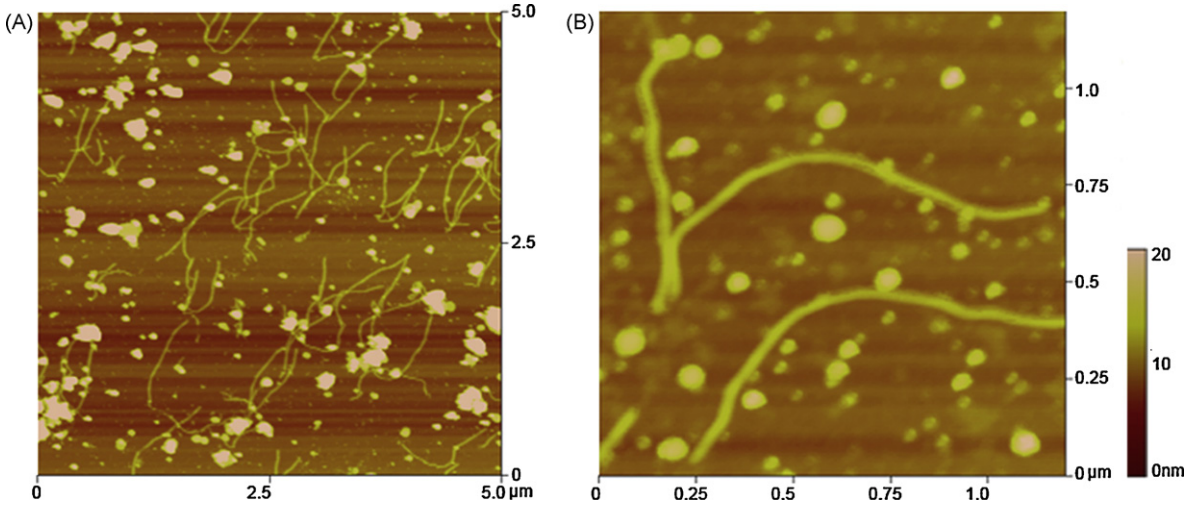

Fig. 4. AFM height images of phage M13KO7 on the mica surface. Image "A" was scanned under 5 μm and 0.8846 Hz and "B" was scanned under 1.2 μm and 1.209 Hz.

Table 2
Data of AFM.

	L (nm)	*Z* (nm)	*S* (nm)
Bio-GP3 on the surface of silicon wafer	98.2 ± 0.14	1.7 ± 0.46	92.6 ± 0.19
Phage M13KO7 captured by antibody GP3	Long filament (approximate 1 μm)	4.42 ± 0.173	39.21 ± 0.30

L is length. *Z* is height. *S* is width. Moleculars of antibody GP3 was approximately oval oblate spheres with a long axis (*L*) and short axis (*S*). The length of phage M13KO7 cannot be measured by AFM for its filamentous shape.

mica surface, where some filamentous phages and particles were seen, which were consistent with previous results (Ji et al., 1998; Nettikadan et al., 2003). The particles in the image were considered to be contaminants in culture solution of phage as discussed in previous studies (Ji et al., 1998; Nettikadan et al., 2003). Furthermore, the phage detection results of BIE (Fig. 5A) was shown in Fig. 5B and C, where no filamentous phage was observed on the area of antibody bio-GP3 (Fig. 5B) and the data were listed in Table 2. The filamentous phage in Fig. 5C was highly consistent with the size and topography shown in Fig. 4. The contaminants particles (shown in Fig. 4) were sparse on the sensing surface with antibody bio-GP3 (shown in Fig. 5C), and cause trivial variation of surface concentration in BIE test. The variation of BIE signal was arising from a large amount of filamentous phage captured by bio-GP3 (Fig. 5C) rather than the sparse contaminants. The molecule of GP3 was similar to an oval oblate sphere (its diameter is about 95 nm and height is about 1.7 nm), whereas phages M13KO7 were long filaments (its length is about 1 μm, height is about 4.42 nm and width is about 39.21 nm). The difference between GP3 and phage M13KO7 were obvious in the two areas, and thus the detection result of BIE was confirmed by AFM.

4. Discussion

We developed a suitable model to realize oriented immobilization of antibodies on the biosensor surface by using avidin. The Fc part of antibody GP3 was quite lysine-rich and the biotin was labeled as GP3 based on covalent bonding between the carboxyl of biotin and ε amide of lysine (Viveros et al., 2006; Zacco et al., 2006). As a result, the Fab parts of GP3 was pointing away from the substrate so that their binding with the domain of antigen was facilitated, which can realize oriented immobilization of ligand for improving the biosensor's sensitivity.

Phage M13KO7 is a kind of virus lodging in *E. coli*, and has no capability to cause human and animal disease. However, its size and structure are similar to the virus causing human disease, and thus the phage detection cannot only ensure experimenter's safety, but also help to develop BIE as a protocol and method of direct virus detection in such aspects as oriented immobilization of ligand, optimization of ligand or related biomolecule (e.g. avidin) and qualitative or quantitative detection. These methods have key significance in applying BIE to disease diagnosis, as well as serving as an earlier and biologic explanation of viruses, e.g. the detection of H5N1 avian influenza virus with BIE was reported recently (Qi et al., 2007). Therefore, this virus detection platform is highly versatile and powerful.

Compared with other capture techniques, two improvements of BIE are demonstrated in the present study. First, multiple detections of numerous samples can be carried out simultaneously by antibody microarray, in other words, an obvious advantage of BIE is its capability to realize parallel analysis, e.g. different batches of purified or natural samples and series of samples prepared by serial dilution could be designed and detected on a single silicon wafer. Second, an important and novel finding of the present study is BIE's ability to detect the whole virus without label, not part of it. AFM has been used to obtain high-resolution images of viruses immobilized on substrates with sensitivity 10^8 pfu/ml (Ji et al., 1998; Kuznetsov et al., 2001), indicating that AFM is competent for scanning and distinguishing positive and negative areas on the substrate of biosensor. Our experiment shows that the whole phage M13KO7 is captured by the sensing surface and the finding of long phage fil-

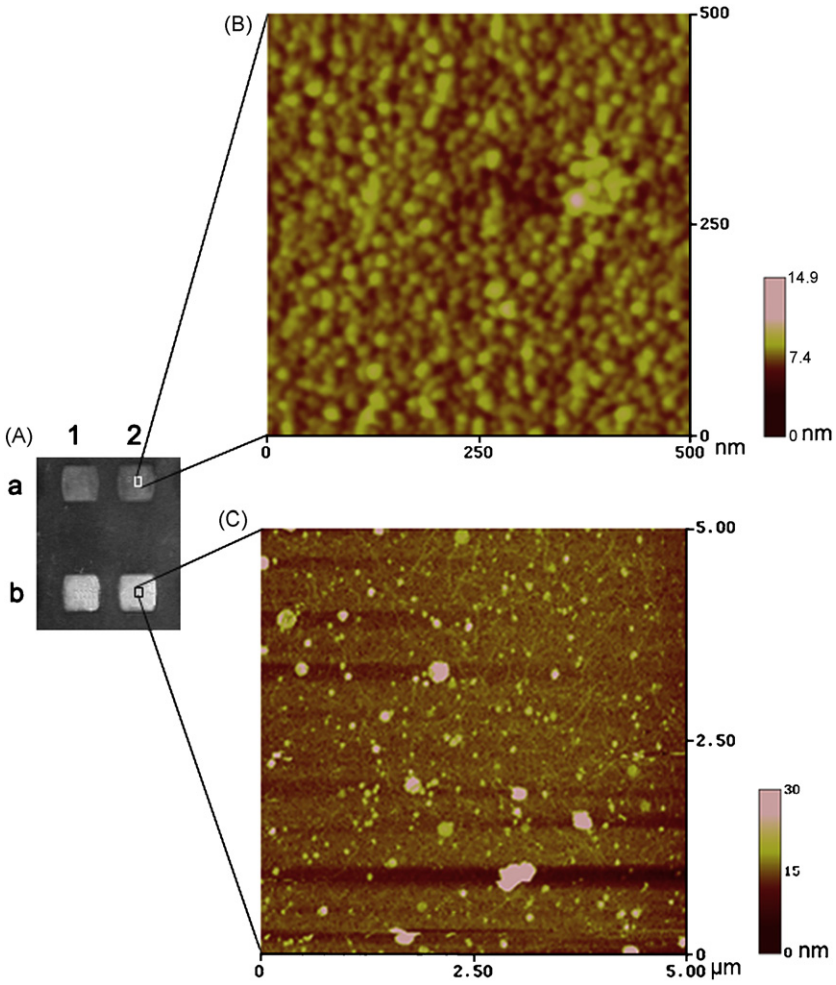

Fig. 5. The result of phage detection of antibody GP3 and phage M13KO7 by using BIE and AFM height images, respectively. (A) The result of BIE: antibody GP3 was immobilized and formed four areas "a1, a2, b1, b2", among which "b1, b2" were adopted to detect phage sample. After BIE detection, the chip is submitted to AFM for scanning: (B) AFM image of scanning under 500 nm on the area containing antibody GP3; (C) AFM image of scanning under 5 μm on the area dedicated for phage detection.

ament is in agreement with previous studies, validating the present results of virus detection. However, some limitation of BIE is also noticeable, in particular, the sensitivity of BIE is still at a low level (10^9 pfu/ml) compared with the sensitivity (10^8 pfu/ml) of AFM and QCM (Nettikadan et al., 2003; Uttenthaler et al., 2001), and thus further improvement in its sensitivity is needed. Another limitation of BIE diagnosis is that it could not offer interactive microcosmic image, however this does not affect its role in virus detection, since rapid clinical diagnosis does not usually require a microcosmic image.

To sum up, this work shows that it is feasible to use BIE as a specific tool to capture virus, which is verified by AFM microscope technique. And thus we might say BIE has a potential for application to direct detection of virus.

Acknowledgments

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