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Label-free microcantilever-based immunosensors for highly sensitive determination of avian influenza virus H9

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Abstract We report on label-free immunosensors for the highly sensitive detection of avian influenza virus. The method makes use of the microcantilevers of an atomic force microscope onto which monoclonal antibodies against avian influenza virus were covalently immobilized. The factors influencing the performance of the resulting immunosensors were optimized by measuring the deflections of the cantilever via optical reflection, and this resulted in low detection limits and a wide analytical range. The differential deflection signals revealed specific antigen binding and their intensity is proportional to the logarithm of the concentrations of the virus in solution. Under optimal conditions, the immunosensors exhibit a linear response in the 7.6 ng mL^{-1} to 76 µg mL^{-1} concentration range of avian influenza virus, and the detection limit is 1.9 ng mL^{-1}.

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Introduction

Biosecurity is one of the foremost challenges facing our global society, through the spread of emerging infectious diseases such as avian influenza, SARS, Hendra and Nipah, and of potential terrorist uses. Consequently biosecurity research has become a fast growing new interdisciplinary field, seeking increasingly to detect, prevent and reduce such threats [1]. The traditional methodologies for the measurement of viruses are based on the "gold standard" which proves the existence of viral antigen by culturing viruses in isolation and detecting them using the method of immunocytology [2]. Two examples of this are PCR and DNA methods which have the advantage of feasibility for determining the microbe and its secretion. These methods are, however demanding in terms of time and the need for enrichment as a pretreatment. Besides this, the DNA method can not be applied in the detection of toxins and extracellular products of infectious agents [3]. Thus, researchers in this field are focusing on the development of accurate and highly sensitive techniques for the measurement of molecules by the combination of conventional immunocytology and microbiology. For example, nested PCR in a magnetically actuated circular closed-loop PCR microchip system has recently been reported [4].

One attractive area for the development of pathogen detection, identification and quantification is the field of biosensor technology [5]. A biosensor is a device for the detection of analyte based on a specific and sensitive biological recognition element in combination with a transducer, with a physicochemical detector component for signal processing. Such biosensors are expected to have significant analytical benefits in such wide-ranging fields as medicine, agriculture, food safety, national security, and in environmental and industrial monitoring [6]. The reliability of an experiment is controlled largely by the "labels" used, which could be enzymes, magnetic beads or fluorophores, where the number of the labels

detected and the target species is supposed to be the same. However, researchers have recognized that the labeling process itself unreliably affects the binding properties of the biomolecules and the percentage yield of coupled species [7], and Amano and Cheng have found that protein targets are more problematic than DNA targets, and that some label-free techniques have potential in the measurement of viruses, for example: surface plasmon resonance (SPR) and quartz-crystal microbalance (QCM). Label-free biosensors such as those used in enzyme-linked immunosorbent assays (ELISA) or DNA sequencing, do not require secondary or tertiary reactions to generate measurable signals, and before analysis, there is no interference from fluorescent or chromogenic tags in such labeling steps [8]. Thus, label-free biosensors are regarded as ideal for fast, direct, continuous and near real-time monitoring of infectious agents [9]. Of these, electromechanical sensors are mass-sensitive, label-free and highly sensitive [10].

A highly promising development of label-free biosensors is that of microcantilever-based biosensors; miniaturized sensors which can be manufactured at low cost, and in large amounts. These nanomechanical sensors have the particular advantage of high sensitivity combined with small area (~100 μm^2), compared with other label-free biosensors such as surface plasmon resonance (SPR) biosensors (~1 mm^2) and quartz crystal microbalances (~1 cm^2) [11]. Microcantilever-based biosensors were first used by Ilic, et al. to detect the immunospecific binding of viruses, which were captured from liquid [12]. Campbell and Mutharasan have demonstrated that microcantilever sensors [13], because of their label-free detection principle, their wide field of application, and their small size, are particularly useful when used as biosensors for the purposes of diagnostic applications, disease monitoring, and research in genomics or proteomics. More recent uses of microcantilever-based biosensors have been for the measurement of cells, viruses, antigen-antibody interactions, DNA hybridization, enzymes and the conformational change of proteins by Goeders, et al. [14]. They have also been applied by Boisen and Thundat as devices for the fast and reliable detection of small amounts of biomolecules in air and in solution [15]. As the amount of substance on the surface of the cantilever varies, cantilever deflection is caused by the resonance and the changes in surface stress, which can be used to measure the interaction of the antigen and the antibody. However, few studies of the clinical application of cantilevers have been reported [16].

This paper is the first to report a microcantilever-based immunosensor that quantitatively detects the infectious avian influenza virus, H9 (AIV), using monoclonal antibodies to AIV as the receptor molecules. The microcantilever was chemically modified by L-cysteine by self-assembly. The amino and sulfhydryl groups of the modifiers were derivatized with glutaraldehyde as a cross-linker. The monoclonal antibodies to AIVs were then immobilized on the corresponding microcantilevers to fabricate the immunosensor. High sensitivity and improved detection range can be obtained by optimizing detection conditions. The differential deflection signals revealed a specific antigen binding and were proportional to the logarithm of the antigen concentrations in solution. We have proved that the micromechanical biosensor allows rapid direct detection of AIV with high sensitivity, without any need for labeling with fluorescent or radioactive molecules. The objective of this work was to broaden applications of microcantilever-based biosensors for pathogen detection and enable the possible future use of the such biosensors in medicine, agriculture, food safety, and environmental monitoring.

Experimental

Materials

Monoclonal antibodies to nuclear protein of AIV were obtained by fusion between marrow cells and spleen cells from BALB c^{-1} mice immunized with H9 subtype AIV from the key open laboratory of preventive veterinary medicine of Jiangsu province in Yangzhou University in China (Yangzhou, China, http://syxy.yzu.edu.cn/). Infectious bursal disease virus (IBDV) and its monoclonal antibodies were also obtained from the above laboratory. Specificity of these monoclonal antibodies was identified by immunofluorescent assay. Bovine serum albumin (BSA, 96–99 %) was obtained from Sigma (USA, www.sigma-aldrich.com). Chromatographically pure ethanol and glutaraldehyde (GA, 25 % solution) were obtained from Aldrich (Shanghai, China, http://sigmaaldrich.bioon.com.cn/). All other chemicals and solvents used were of analytical grade and were used as received. The phosphate buffer solution (pH 7.0, 20 °C) was prepared by mixing 0.02 mol L^{-1} disodium hydrogen phosphate solution and 0.02 mol L^{-1} sodium dihydrogen phosphate solution, and was used as the default buffer for all our experiments. The blocking solution was prepared by dissolving 50 μg BSA into 10 mL phosphate buffer solution. All solutions were stored at 4 °C.

Apparatus

All responses of the present immunosensor were recorded using an atomic force microscope (AFM, Digital Instruments, Multi-mode Nanoscope III (a) scanning probe microscope, USA) equipped with a diode laser, a spatial filtering and focusing system, and an in-house-built position-sensitive optical detector. The micro-fabricated cantilever (193×20× 0.6 μm) was made of silicon nitride, and was commercially available. It is V-shaped, fixed to a large substrate chip, and coated on one side with a thin layer of gold (25 nm). The

silicon nitride microcantilever was fixed onto a silicon chip, which was then bonded inside a 50 μL volume Teflon flow cell. A collimated laser light beam from a low power laser diode was focused on the free end of the cantilever to monitor the deflection by a position sensitive detector (PSD). The experimental system was located on a vibration isolation table. SEM measurements were conducted on an S-4800 II TESEM from Hitachi High-Technologies Corporation (Japan) at an accelerating voltage of 15 kV.

Fabrication of the avian influenza virus immunosensor

Firstly, the gold-coated microcantilever was immersed in freshly prepared Piranha solution (70 % H_2SO_4 and 30 % H_2O_2) in order to clean the surfaces, followed by thorough rinsing with deionized water, ethanol, and phosphate buffer solution, sequentially. For the immobilization method used in our work, we referred to the conventional and optimized approach for antibody immobilization onto an Au electrode as an electrochemical immunosensor [17]. For the detection of AIV, the microcantilever was chemically modified by L-cysteine, for which it was carefully grasped by using thin-tipped tweezers, and one side was kept in contact with 2 mmol L^{-1} of L-cysteine aqueous solution for 24 h, in order to self-assemble a monolayer on the surface which had been spray-coated with gold. Thus, L-cysteine was grafted onto the gold surfaces of the cantilevers by the Au-S bond. The amino groups were then derivatized with the cross-linker by dipping the cantilevers in a 5 % (w/V) solution of GA for 4 h. After rinsing in deionized water, the treated cantilever device was incubated in 0.27 μg mL^{-1} AIV monoclonal antibody solution for the detection of AIV overnight at 4 °C, and then it was rinsed in phosphate buffer solution for 5 min. BSA was used as a blocking agent to prevent non-specific binding of other contaminants onto the cantilevers. In order to achieve this, the cantilever was immersed in a solution of 5 μg mL^{-1} BSA in the phosphate buffer solution at 37 °C for 4 h. The immunosensor based on the microcantilever was thus constructed for the subsequent deflection measurements for sensing biomolecules with an immunoreaction. The functionalized surface was freshly prepared for each experiment.

Experimental measurements

An atomic force microscope was used to obtain the detection information (a schematic diagram of the microcantilever immunosensor for virus detection is given in Electronic Supplementary Material (ESM), Section S1). A gold-coated microcantilever, which was modified with a self-assembled monolayer of L-cysteine to detect AIV, served as the surface for immobilizing capture molecules (monoclonal antibodies). The cantilever was immersed in the phosphate buffer solution containing the monoclonal antibody, leaving antigen binding

sites available with minimal steric hindrance to binding of the target analyte. The residual sites on the surface were blocked with BSA. Once the reaction between receptor molecules and specific biomolecules has occurred, the surface stress of the cantilever changes and leads to cantilever deflection, which can be measured in nanometers, so the deflection data indicate the specific biomolecular reaction. The microcantilever deflection measurements were carried out by using the optical beam deflection technique as described below: the bending of the microcantilever was detected by aligning the focused light from a low power laser diode onto the free tip of the microcantilever and monitoring the displacements of the reflected laser light with a position sensitive detector. The output of the data was displayed and recorded under deflection mode of the AFM. Data were collected at 0.1 Hz to generate graphs. Here, phosphate buffer solution was used as a background solution and a thermal environment of a constant 37 °C was maintained. Baselines were recorded before any injection of antigen solution. Firstly, a micro-syringe was used to inject 50 μL phosphate buffer solution into a flow cell, which is an accessory of this AFM. The stable deflection signal recorded indicated that the microcantilever was in balance in the flow cell (see figure from AFM scanning in Electronic Supplementary Material, Section S2). This process usually lasted for 30 min. Following this, 100 μL of antigen solution was transferred into the fluid cell so that the phosphate buffer solution in the fluid cell with a volume of 50 μL could immediately be completely replaced by antigen solution.

Results and discussion

Mechanism of the microcantilever-based immunosensor

The antigens bind to the immobilized antibodies in the biological coating, with a resulting change in surface stress, generating a difference in the surface stress between the opposite sides of the microcantilever, which induces a permanent bending. Detection is based on the change in the deflection response before and after the antigen–antibody interaction. The deflection of the microcantilever, indicating the presence of the target analyte, is monitored and recognized by an optical lever method. The microcantilever's motion is recorded during the entire reaction by monitoring the spot of light which it reflects. The displacement of the reflected laser light is recorded with a position sensitive detector [18].

AFM characterization of microcantilever surface

Immobilization of receptors on the sensor surface is of central importance to the design of a successful biosensor assay. It was an important precondition that the microcantilever's sensing

layer was as uniformly covered with monoclonal antibodies as possible. Figure 1 shows the topographic features of the microelement surface before and after the modification, and the results from the immunoreaction are revealed by the corresponding AFM and SEM images. Figure 1(a) and (A) show an image of the blank microcantilever. After the microcantilever was modified with the sensing layer and blocked with BSA, there were obvious differences compared with the blank microcantilever, as shown in Fig. 1(b) and (B). The increase in the surface roughness may depend on covalent linking of the AIV monoclonal antibodies to the L-cysteine on the surface in a three-dimensional antibody network, with the RMS roughness increasing to 49.388 nm. A random inhomogeneous cloud-like structure with

more globular aggregates can now be seen. It demonstrates that antigen molecules were well-linked to the monoclonal antibodies. Figure 1(c) and (C) show the morphology of the microcantilever immobilized by antibodies, with 101.71 nm of roughness after incubation in AIV monoclonal antibody solution for 4 h at 37 °C. These AFM images provide information on the actual microcantilever surface, indicating that the antibody-antigen special interaction occurred successfully.

Deflection responses of the immunosensors

Figure 2(a) shows the deflection responses of the immunosensor to sequential injection of phosphate buffer

Fig. 1 AFM and SEM images of microcantilever surfaces: blank microcantilever substrate (a and A); after immobilization of AIV antibodies and blocking by BSA (b and B); after incubation in AIV solution for 4 h (c and C); A tapping mode was selected and a micro-fabricated silicon cantilever with a bending spring constant of 20–80 N m^{-1} and a resonance frequency of 229–287 kHz was used for imaging at a scan rate of 1.0 Hz. Other conditions are the same as in the Experimental part
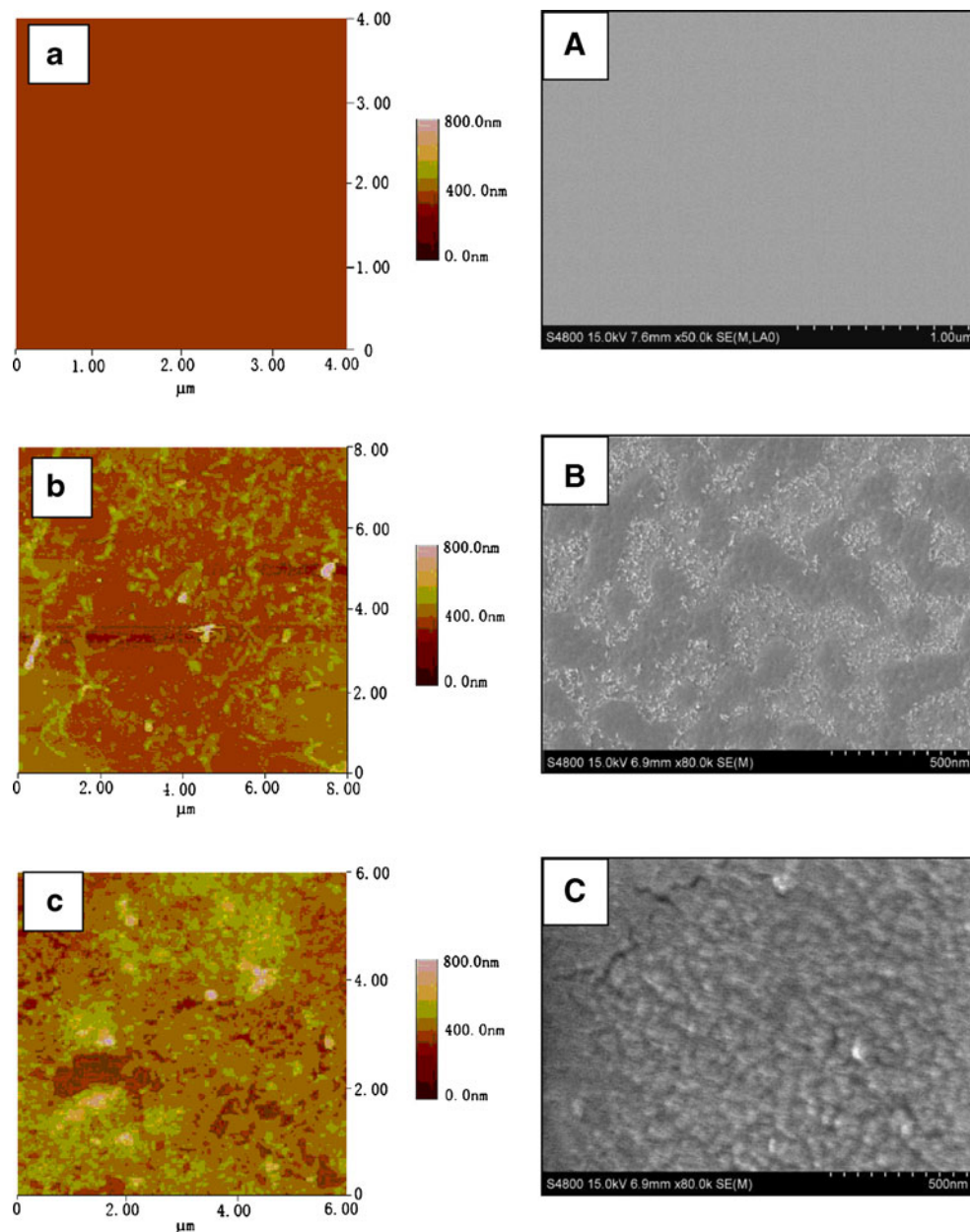
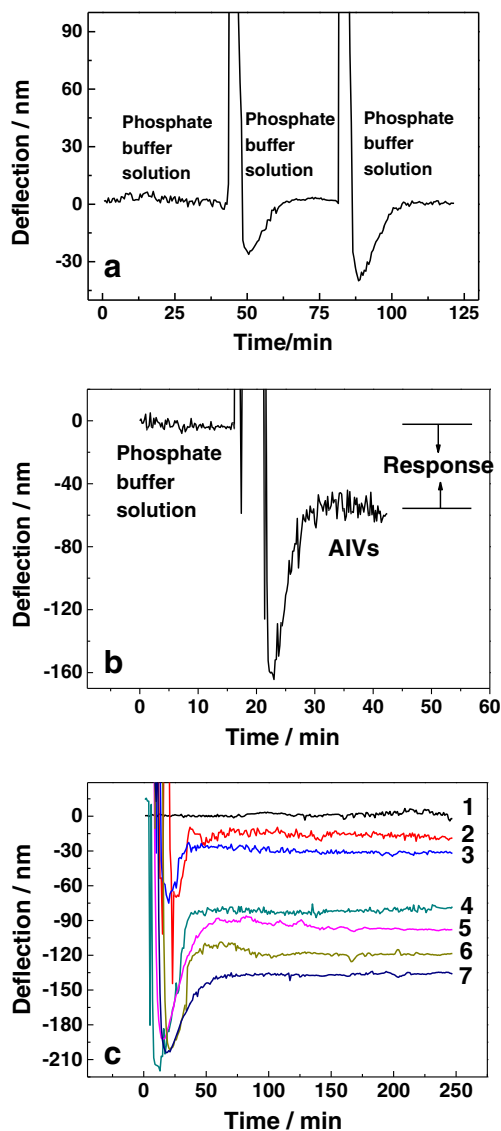**Fig. 2** Deflection responses of immunosensors: Three sequential injections of the phosphate buffer solution (0.02 mol L^{-1} pH=7.0) (**a**); after injection of 7.0×10^{-5} μg mL^{-1} AIV (**b**); sequential injections of AIV, 1-Blank solution (phosphate buffer solution), 2–7.6×10^{-6} μg mL^{-1}, 3–3.8×10^{-5} μg mL^{-1}, 4–3.8×10^{-4} μg mL^{-1}, 5–7.6×10^{-3} μg mL^{-1}, 6–3.8×10^{-2} μg mL^{-1}, and 7–7.6×10^{-2} μg mL^{-1} (**c**). Other conditions are the same as in the Experimental part

solution. The cantilever deflection goes back to the exact deflection as before the injection, indicating that there was no deflection for the immunosensor, and the responses were stable after 2 h in a blank solution, with the exception of the abrupt peaks caused by sudden injection-induced impulsion. However, the deflection response rapidly returned to baseline within a few minutes. Figure 2(b) shows the obvious deflection response of the immunosensor to the AIV. Figure 2(c) shows sequential injections of AIV solutions with different concentrations. The higher the concentration of AIV was, the greater the deflection of the immunosensor. This was the basis of the quantitative determination of the viruses.

Optimization of immunosensor responses

We examined the effect of temperature on the immunoreaction. For this measurement, the built-in temperature controller was set to 37 °C. We observed that there was a significant change in sensitivity between 25 and 50 °C in incubation and immunoreaction, and the most sensitive response was obtained at 37 °C when the other conditions were same. The phosphate buffer solution acidity for immunoreactions was investigated. Experimental results indicated the deflection response to detect AIV with the immunosensor immobilized by monoclonal AIV antibodies at varying pH values of phosphate buffer solution. So, phosphate buffer solution at pH 7.0 was selected to detect AIV.

The quantity of antibodies immobilized on the immunosensor is also a key factor in the sensitivity of the immunosensor. When we changed the antibody concentration in the immobilizing solution and examined the deflection response of the immunosensor, different responses were observed. Figure 3 shows the deflection response of the AIV immunosensor to 7.6×10^{-6} μg mL^{-1} AIV. The response increased with increasing AIV antibody concentration in the immobilization solution in the range from 0.013 to 0.25 μg mL^{-1}, and the response became constant once AIV antibody concentration exceeded 0.25 μg mL^{-1}, owing to a saturation of the interaction between AIV antibodies and GA. So, a concentration of 0.27 μg mL^{-1} AIV antibodies was selected as optimal in the immobilization solution. At the same time, the contact time between the antibodies and the GA was evaluated according to the respective deflection response of the immunosensor, and 24 h was selected as the optimal interaction time in order to ensure saturation of the interaction between the antibodies and the GA. The immobilized antibodies were found to have a stable active lifetime of up to 7 weeks.
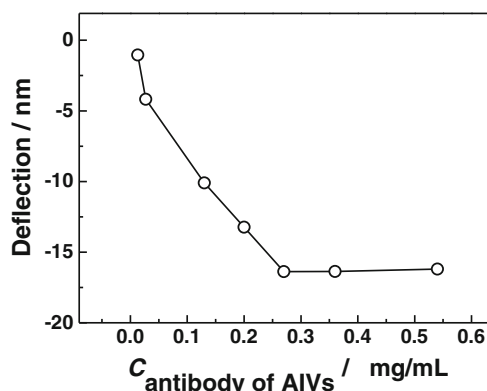

Fig. 3 The influences of antibody concentration in immobilization solutions on the response of the immunosensor: antibodies to AIV in pH 7.0 phosphate buffer solution. Interaction time: 24 h at 37 °C. Other conditions are the same as in the Experimental part
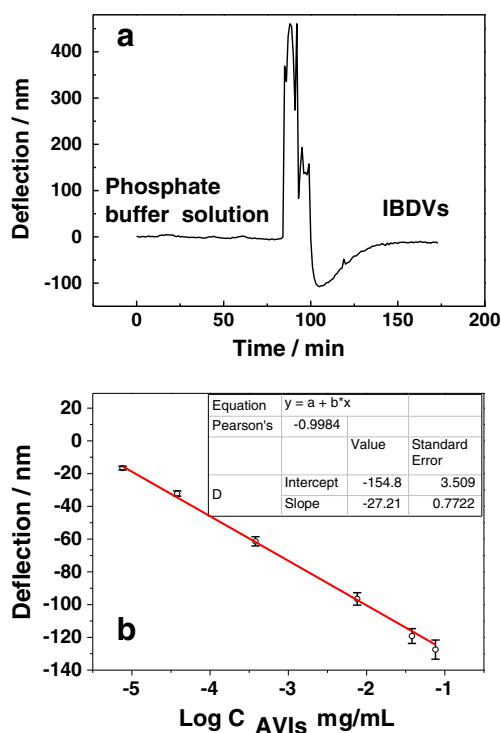
Fig. 4 Deflection response of immunosensor immobilized by monoclonal AIV antibodies to IBDV solution (**a**). Plots show the linear relationships for AIV analysis (**b**)

Immunoreaction specificity

The interaction between an antibody and an antigen is known to be a very specific reaction. Such specific molecular recognition of antigens by antibodies has been exploited in immunosensors to develop highly selective detection of proteins [19]. The immunoreaction specificity of the immunosensor was investigated by recording the response of our immunosensor to another antigen. Figure 4(a) shows the response of the immunosensor immobilized by monoclonal AIV antibodies to IBDV solution, and the cantilever deflection

goes back to the exact deflection as before the injection. The above experimental results indicated that the immunosensor in this study has immunoreaction specificity and good selectivity for determination of analytes. An antigen-specific antibody fits its unique antigen in a highly specific manner. Antibody-based sensors offer the potential for rapid analysis, which may be used for on-site measurements.

Calibration plots

After the above optimizations for the determination of AIV, a good linear relationship between the deflection and the logarithm of the concentration of AIV in the concentration range from 7.6 ng mL^{-1} to 76 μg mL^{-1} was obtained (Fig. 4(b)). The relationship between the deflection responses and the concentration of the AIV solution is shown as a regression equation of D (nm) = − 154.8–27.21 log C_{AIVs} (μg mL^{-1}), with a correlation co-efficient of 0.9984 (*n*= 6). The threshold for the positive detection was set as background (blank) signal+ 3×noise (standard deviation) and the detection limit of the immunosensor was determined as 1.9 ng mL^{-1}. Table 1 is provided for the performance comparisons between our microcantilever-based immunosensors and other label-free detection methods reported in the literature on the detection of the influenza virus, including QCM, SPR, LSPR, electrochemical sensor and so on. The advantage of microcantilever-based sensors is their small size, combined with their performance, which is as good as that of SPR or QCM. Both are well established, portable, and commercially available for a reasonable price. The detection sensitivity of our sensor as a mass sensor is higher than those of conventional QCM methods. Because our microcantilever sensor is based on deflection detection by laser and so uses optical rather than acoustic waves or frequency, our microcantilever sensor, like surface plasmon resonance devices, is ultimately capable of a higher precision. But for SPR, any artifactual refractive index (RI)

Table 1 The performance comparisons between our microcantilever-based immunosensors and other label-free detection methods reported in literatures

Detection method	Target virus	Recognition element	Estimated measurement time	Detection range	Reference
QCM	AIV H5N1	pAb- nanobeads	2 h	0.128 to 12.8 HAU	[20]
QCM	Human influenza A and B viruses	pAb–Au NPs	1 h	25–2.5×10^4 μg mL^{-1}	[21]
QCM	SARS corona virus	pAb	2 h	0.6–4 μg mL^{-1}	[22]
SPR	AIV H5N1	DNA	1.5 h	0.128–1.28 HAU	[23]
LSPR	AIV H5N1	pAb	–	30 pg mL^{-1} ~	[24]
Quantum dot probe	AIV H5	mAb	30 min	0.27–12 ng mL^{-1}	[25]
ITO TFTs	AIV H5N1	mAb	–	5–5×10^3 ng mL^{-1}	[26]
Electrochemical impedance sensor	AIV H5N1	pAb	2 h	10^3 EID$_{50}$ mL^{-1}	[27]
Microcantilever sensor	AIV H9	mAb	30 min	7.6–7.6×10^3 ng mL^{-1}	This work

pAb polyclonal antibody; mAb monoclonal antibody; DNA deoxyribonucleic acid

change other than that due to the interaction can also give a signal. In some ways, the present sensor might be superior to SPR methods for a wide linear range and detection time. We will devote ourselves to miniaturization of this detection system with integrated chips and without the use of an AFM in future work, and promote its promising application in biomedical science.

From our experimental results, the values of association constant k_a and dissociation constant k_d were found to be $2.47 \times 10^4 \, \mathrm{M^{-1} \, s^{-1}}$ and $4.16 \times 10^{-4} \, \mathrm{s^{-1}}$, respectively. Therefore, the corresponding equilibrium dissociation constant K_D is 5.94×10^{-9} M, indicating strong binding between the monoclonal AIV antibodies and AIVs. The KD is near the value 4.65×10^{-9} M as determined by SPR for AIV H5N1 [28].

Reutilization of the microcantilever used

The microcantilever immunosensor used was immersed in Piranha solution (70 % H_2SO_4 and 30 % H_2O_2) for 30 min, and then rinsed with water three times. In the above simple procedure, antigens, antibodies and modifiers were removed completely, and the cleaned microcantilever could be used again for the fabrication of another immunosensor. This reutilization can lower the cost of analysis.

Conclusions

We have taken advantage of bio-chemically induced surface stress to directly and specifically transduce molecular recognition into nano-mechanical responses in a microcantilever sensor to successfully detect AIV. This is achieved by immobilizing antibodies on one side of the cantilever and then detecting the mechanical bending induced by antigen–antibody specific binding in a liquid environment. A major advantage of this type of direct transduction is elimination of external optical displacement measurement systems and the requirement that the molecules under investigation be labeled, for example, with fluorescence or radioactive tags. The microcantilever system can provide a low cost, high sensitivity, high throughput, and attractive alternative platform for pathogen detection in the future. This work should broaden applications of microcantilever-based biosensors for pathogen detection and enable the possible future use of such biosensors in medicine, agriculture, food safety, and environmental monitoring.

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