Immunosensor Based on Antibody-Functionalized MoS2 for Rapid Detection of Avian Coronavirus on Cotton Thread

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Abstract—Infectious bronchitis virus (IBV), an avian coronavirus, significantly affects the performance of both the egg-laying and meat-type birds causing the foremost of economic loss in poultry industry. This paper aims to develop a rapid, low-cost, and sensitive biosensor for IBV detection by using molybdenum disulfide (MoS2). MoS2 is a 2-D nanosheet which has strong high fluorescence-quenching ability when applied to a dye-labeled antibody (Ab). In this paper, we developed an Ab-functionalized MoS2-based fluorescent immunosensor, which utilized the fluorescence resonance energy transfer (FRET) between the MoS2 and fluorescence dye during the Ab–antigen interaction. The assay was performed on a low-cost cotton thread-based microfluidic platform due to the good wicking property and flexibility. Upon the optimization of assay conditions, the immunosensor demonstrated remarkable sensitivity of \(4.6 \times 10^2\) EID50 per mL and specificity with a dynamic linear response range of \(10^2–10^6\) EID50 per mL for IBV standard solutions. The developed immunoassay successfully detected the IBV spiked chicken serum with satisfactory results. The foregoing presents its potential application for on-farm detection.

Index Terms—Infectious bronchitis virus, molybdenum disulfide, FRET, immunosensor, cotton thread.

I. INTRODUCTION

Coronaviruses are enveloped, positive-strand RNA viruses of birds and mammals including humans. As a representative of the Gamma-coronavirus genus, the avian coronavirus (AvCoV) infectious bronchitis virus (IBV) may cause highly contagious respiratory disease in chickens and other galliforme birds [1]. IBV spread fast between individuals may cause a morbidity rate of 100% in bird populations which have not been vaccinated [2]. Chickens infected with IBV exhibit the symptoms of mild respiratory such as coughing, gasping, rales, and nasal discharge, and appearing depressed, or severe kidney and oviduct disease [3], [4], which may result in a decrease in egg production or quality. Development of affordable and highly sensitive detection methods for rapid monitoring and screening of IBV is extremely important, especially in less-developed countries. The enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) test have now been used as gold standard for nucleic acid biomarker diagnostics. However, conventional ELISA can only work on a higher concentration of target molecules hence makes the approach not quite suitable for highly sensitive detection, while PCR usually requires expensive reagents and equipment as well as skilled personnel for the complicated process [5]. All these limitations restrict their applications in on-site detection.

Nanomaterials have become powerful element in constructing new biosensors due to their unique optical, electronic and catalytic properties [6]–[8]. Recently, transition metal dichalcogenides (TMDs) have gained world-wide attention as a group of 2D layered nanomaterials analogous to graphene owing to their excellent optoelectronics, nanoelectronics, and energy-harvesting properties. TMDs have been widely used in many research including DNA detection, transistors, photodetectors and photovoltaic devices [9], [10]. Molybdenum disulfide (MoS2) is an emerging material and one of such TMDs that can be synthesized in large scale and directly dispersed in aqueous solution and no treatment of surfactants or oxidation is required [11], [12]. Moreover, a higher fluorescence-quenching ability than graphene and the hydrophobicity property of surface make MoS2 become promising material in biosensing platforms and finding various applications [13]–[18]. The hydrophobicity of the MoS2 surface is a key enabling feature, because it enables strong affinity of protein-surface adsorption [11]. Kong et al. [11] made an aptamer-functionalized MoS2 biosensor by using the high fluorescence-quenching ability between MoS2 and dye-labeled single-stranded DNA probe for prostate specific antigen detection. High sensitivity and high selectivity with a detection limit for the PSA of 0.2 ng/mL were achieved. Tuteja et al. [18] reported a MoS2-based electrochemical immunosensor for detecting \(\beta\)-hydroxybutyrate, which is biomarker of subclinical ketois. The immunosensor is based on the immunodetection of the anti-\(\beta\)-HBA antibodies immobilized on the MoS2-modified electrodes and \(\beta\)-HBA antigen. Geldert et al. [19] developed fluorescence resonance energy transfer (FRET)-based MoS2 aptasensor for the detection of the malarial biomarker Plasmodium lactate dehydrogenase (pLDH). Zhang et al. [20] demonstrated a sandwich electrochemiluminescence immunosensor based on MoS2 for alpha fetal protein detection. MoS2 nanosheets surface was modified using polyethylenimine (PEI) polymer

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and gold nanoparticles. The immunosensor was able to analyze AFP in real human serum samples with limit of detection of $1.0 \times 10^{-5}$ ng/mL. These research demonstrate the huge protetial of MoS$_2$ in biosensor applications.

The thread-based microfluidics is promising alternative to conventional microfluidic systems due to its many special characters. The thread-based biomedical devices are low cost and broadly available, flexible, easy to handle, lightweight, easy to manipulate and facilitate to transported or stored in any forms. In addition, the wicking properties of thread enable the low volumes of sample solution through it efficiently. All above mentioned makes thread-based microfluidics an attractive matrix for the fabrication of low-cost and low-volume microfluidic diagnostic devices for handheld on-site diagnosis applications [21]–[23].

Here, we aimed at developing a biosensor for rapid detection of IBV. We developed a single-step immunosensor on cotton thread based on antibody-functionalized MoS$_2$ for the detection of IBV. The principle is based on a homogeneous FRET immunoassay, the sensing mechanism is shown in Fig. 1. The distinct quenching property of MoS$_2$ on the fluorophore during the antigen-antibody reaction was adopted. The antibody (Ab) probes are modified with fluorescent dye labelling (dyed-IBV-Ab) and MoS$_2$(MoS$_2$-IBV-Ab bioconjugates), respectively. In the presence of IBV, both the dyed-IBV-Ab and the MoS$_2$-IBV-Ab bioconjugates will specifically bind with the target IBV due to the antigen-antibody reaction. After binding, the fluorescence of the dyed-IBV-Ab probe is largely quenched owing to the transfer of electrons or energy between the closely connected dye molecules and the MoS$_2$. In the absence of IBV, the fluorescence of the dyed-IBV-Ab probe will not change. Therefore, the concentration of the IBV in the chicken blood sample can be quantitatively determined by analysis the fluorescence intensity in an assay.

**II. IMMUNOSENSOR PRINCIPLE AND EXPERIMENT**

**A. Materials**

MoS$_2$ nano-sheet dispersion, ovine serum albumin (lyophilized powder, $\geq$96%), hydrogen peroxide (H$_2$O$_2$), poly(L-lysine) (PLL), chicken serum and all other mentioned chemicals and solvents were purchased from Sigma-Aldrich (Oakville, ON, Canada). Anti-infectious bronchitis virus (Massachusetts) (IBV) was purchased MyBioSource, Inc. (San Diego, CA, USA). Infectious bronchitis virus, low pathogenic avian influenza virus A H4N6 and H9N2 were kindly provided by our collaborator (Ontario Veterinary College, Canada) and the detailed culture procedure can be found elsewhere [24]. Briefly, isolate of IBV was propagated in embryonated specific pathogen free (SPF) chicken eggs followed by the titration determined by the method of Reed and Muench. The viral titer of the stock solution was $1 \times 10^6$ EID$_{50}$ per mL (egg infectious dose 50%). Avian influenza virus A H4N6 (Avian influenza virus A/Duck/Czech/56 (H4N6)) was propagated in 11-day-old embryonated chicken eggs by inoculation into the allantoic cavity [25]. Inactivated Avian influenza virus A H9N2 (A/Turkey/Ontario/1/66) was propagated in 10-day-old embryonated SPF chicken eggs followed by inactivating with formalin (final concentration 0.02%) for 72 h at 37 °C [26]. Alexa Fluor 488 Antibody Labeling Kit was purchased from Life Technologies Inc. (Burlington, ON, Canada). Chicken whole blood (Cat. no. IR1-080N) was obtained from Innovation Research, Michigan, USA. Milli-Q water (18.2 MΩ) was used in all experiments.

**B. Preparation of Dyed-IBV-Ab**

The labelling of IBV Ab with fluorescent dye is conducted by using the Alexa Fluor 488 Antibody Labeling Kit. Briefly, 1.0 mg/mL IBV Ab was mixed with 1 M sodium bicarbonate solution. Then 100 μL of the mixture was added into the vial of Alexa FLuo dye followed by an incubation of 1 h at room temperature. The labeled antibody was then obtained via purification using a purification column with resin bed and centrifuged at 1100×g for 5 min. The labeled antibody was stored at 4 °C for further use.

**C. Preparation of MoS$_2$-IBV-Ab Bioconjugates**

The MoS$_2$ acted as a nanoplatform to adsorb IBV Ab and was further employed as a biological probe. The MoS$_2$ (0.5 mg/mL) dispersion in aqueous was first concentrated by centrifugation at 8000 rpm for 30 min to remove the surfactant. The MoS$_2$ was resuspended in Milli-Q water and sonicated for 1 h to provide a homogeneous solution. Aqueous poly(L-lysine) solution was added to the resulting MoS$_2$ at a concentration of 1 mg/mL and stirred thoroughly for 1 h followed by overnight incubation at 4 °C. The poly(L-lysine) backbone is electrostatically adsorbed to MoS$_2$ surface. The PLL-MoS$_2$ mixture was purified and concentrated by centrifugation three times at 8000 rpm for 30 min to remove the solvent and resuspended in PBS. IBV Ab was then added at a desired concentration into the obtained mixture followed by overnight incubation at 4 °C to produce the MoS$_2$-IBV-Ab bioconjugates. With the chemical modification by poly(L-lysine), the antibodies can be bind to MoS$_2$ via electrostatic and covalent interactions [27]. The resulting mixture was purified at 5000 rpm at 4 °C and resuspended in PBS with desired concentrations for further use.
orescent imaging \((\text{Ex/ Em}=488 \text{ nm/519 nm})\) was taken on a fluorescence microscope equipped with a charge-coupled device camera. The intensity of the control zone and the background zone were used for calculation. Fluorescence images were converted into a numerical response using ImageJ software, a region of interest on the thread was drawn on the thread to perform the measurement.

**F. Operational Procedure**

In a typical assay, a small volume \((30 \mu L)\) of the fluorescence probe (dyed-IBV-Ab) and sample solution were added into the inlets of functionalized threads, respectively. An incubation time of \(0\sim12\) min at room temperature was then given to ensure the antigen-antibody reaction. After incubation, the fluorescence measurements were taken and recorded to analyze the change in fluorescence intensity using a fluorescence microscope equipped with a charge-coupled device camera. The intensity of the control zone and the background zone were used for calculation. Fluorescence images were converted into a numerical response using ImageJ software, a region of interest on the thread was drawn on the thread to perform the measurement.

**G. Validation and Optimization of Detection Conditions**

The validation and optimization of the sensing mechanism was performed by fluorescent spectra analysis on a microplate reader. The effect of the concentrations of dyed-IBV-Ab \((10 \mu g/mL, 20 \mu g/mL\) and \(30 \mu g/mL)\), MoS\(_2\)-IBV-Ab bioconjugates and incubation time on the fluorescence quenching were studied. Ten microliter of MoS\(_2\)-IBV-Ab, sample and dyed-IBV-Ab were sequentially added in the microwell followed by fluorescence intensity analysis. All experiments were conducted in triplicate.

**H. Immunosensing of IBV in Chicken Whole Blood and Specificity**

The performance of the presented immunosensor for detecting real blood sample was validated. IBV of various concentration \((1, 5, 10 \times 10^4\) EID\(_{50}\) per mL) was spiked in the chicken whole blood and loaded into the sensor. The average fluorescence intensity is then calculated to determine the sample concentration. To evaluate the specificity of the presented immunosensor, avian influenza A H4N6 and H9N2 were detected. The fluorescence signals with these non-specific analytes were recorded.

**III. RESULTS AND DISCUSSION**

**A. Characterization**

The structure and morphology of the MoS\(_2\) dispersion was investigated with the use of TEM (Fig. 3 (a)). TEM of the MoS\(_2\) nanosheets emphasized the uniformly sized nano MoS\(_2\) with a sheet-like morphology. The size distribution and zeta \((\zeta)\) potential of the MoS\(_2\), PLL-MoS\(_2\) dispersion and MoS\(_2\)-IBV-Ab bioconjugates were characterized to evaluate the binding by dynamic light scattering (DLS) system (Malvern Zetasizer Nano ZS, UK). As shown in Fig. 3 (b), the size distribution of MoS\(_2\), PLL-MoS\(_2\) dispersion and MoS\(_2\)-IBV-Ab bioconjugates are...
The optical properties of MoS\(_2\) indicate the bind of the antibody and moderate dispersion of the antibody conjugated nanoparticles respectively, which were investigated as well as the concentration of the dyed-IBV-Ab. Therefore, the optimized concentration of the dyed-IBV-Ab was investigated by carried out the detection with a series of concentration, 3 \(\mu\)g/mL, 6 \(\mu\)g/mL, 9 \(\mu\)g/mL and 12 \(\mu\)g/mL. The result with a reaction time of 10 min is shown in Fig. 4 (b), the quenching effect increases as the increase of the concentration of the dyed-IBV-Ab while becoming “constant” when it goes above to 9 \(\mu\)g/mL. Therefore, the reaction time duration was also studied, an experiment was carried out by detecting the IBV standard of 1 \(\times\) 10\(^5\) (IBV5) EID\(_{50}\) per mL using the optimized concentrations of MoS\(_2\)-IBV-Ab and dyed-IBV-Ab, 0.2 mg/mL, 0.3 mg/mL, 0.1 mg/mL and 0.2 mg/mL, respectively. Time duration of 1 min, 5 min, 10 min and 20 min were investigated, the response of the sensing method is shown in Fig. 4 (c). The result shows that 10 min is more than sufficient to complete the quenching. Three triplicates were carried out for each data point and then the average of three independent measurements was calculated, the error bars indicate the standard deviation of the mean (n = 3). Under the optimized settings, fluorescence analysis for a series of IBV standard solution (from 0 to 10\(^6\) EID\(_{50}\) per mL, in PBS buffer of pH 7.4) was conducted, the result of which is shown in Fig. 4 (d). The result highlights a decrease in fluorescence intensity with the increased IBV concentration.

**C. Detection of IBV on Cotton Thread**

With the optimization settings as demonstrated in previous section, quantitative analysis of IBV was carried out on the cotton thread. The transportation of fluidic was investigated firstly by using food color and it was observed that the fluid could reach the test zones within 1 min due to the capillary

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**Fig. 3.** (a) TEM image of MoS\(_2\). (b) Particle size distribution of pure MoS\(_2\), PLL-MoS\(_2\), MoS\(_2\)-IBV-Ab by DLS. The mean hydration diameter of the MoS\(_2\), PLL-MoS\(_2\), MoS\(_2\)-IBV-Ab are 21.9 nm and 47.9 nm, respectively. (c) UV-Vis absorption spectrum for MoS\(_2\) dispersion in DI water, PLL-MoS\(_2\), MoS\(_2\)-IBV-Ab and pure IBV Ab. (d) Fluorescence spectra of the dyed-IBV-Ab.

**Fig. 4.** Sensing mechanism optimization and validation: (a) effect of concentrations of MoS\(_2\)-IBV-Ab on the biosensor; (b) effect of concentrations of dyed-IBV-Ab (3 \(\mu\)g/mL, 6 \(\mu\)g/mL, 9 \(\mu\)g/mL and 12 \(\mu\)g/mL) on the biosensor; (c) effect of reaction time duration (1 min, 5 min, 10 min, 15 min and 20 min) on the biosensor; and (d) illustrative fluorescent spectra of the biosensor tested with multiple concentration of IBV standards ranging from 10\(^{−}\) to 10\(^{6}\) EID\(_{50}\) per mL.
wicking. In addition, a time duration of 6 min was found to be sufficient for a complete reaction, as shown in Fig. 5 (a). The reaction time is slightly shorter that conducted in microplate. This may be attributed to the faster kinetics due to the better mixing effect on the thread. A wide range of IBV standard solution was detected. The IBV standard solution ranging from $0 \sim 10^6$ EID$_{50}$ per mL was analyzed by the presented thread immunosensor to obtain the standard curve and calculate the limit of detection. A linear fit of the obtained relative fluorescence intensity difference with respect to varying concentration of IBV standards is plotted in Fig. 5 (b). The values denote average relative fluorescence intensity difference ($n = 3$) compared to negative control with standard deviation as error bars. It is shown that a distinct change presents starting from $10^2$ EID$_{50}$ per mL with a correlation coefficient ($R^2$) of 0.9828. The limit of detection calculated based on $3\sigma$ of the blank is $4.6 \times 10^2$ EID$_{50}$ per mL. The different fluorescence responses of the test zones on the cotton thread upon the detection of various concentrations of IBV standard solution are shown in Fig. 5 (c). It is clearly seen that the fluorescence of the dyed-IBV-Ab was significantly quenched for all concentrations of IBV standard solution. With the concentration of IBV increases, the fluorescence is quenched more because more “sandwich” complex of dyed-IBV-Ab / IBV / MoS$_2$-IBV-Ab are formed, resulting in largely quenched owing to the transfer of electrons or energy between the closely connected dye molecules and the MoS$_2$.

**D. Accuracy of the Biosensor**

The specificity of the developed cotton thread biosensor was evaluated against non-specific virus, avian influenza A H4N6 (100 HAU/50 μL) and H9N2 (100 HAU/50 μL). As shown in Fig. 5 (d), no distinguishable singles were obtained in the presence of the introduced interferents, which indicates that the highly specific towards to IBV.

ELISA was carried out side by side to validate the accuracy of the biosensor. Briefly, a ninety-six-well Maxisorp microtitre plate (Life Technologies Inc., Burlington, ON, Canada) was coated with the IBV Ab at 10 μg/mL diluted in filtered PBS (pH 7.4), and incubated overnight at 4 °C. The wells were washed with 0.05% Tween-20 (1× PBS, pH 7.4) three times followed by being blocked with 200 μL of 3% BSA in PBS at room temperature for 2 h. After washing for three times, 100 μL of standards and spiked chicken blood were added, incubated for 1 h at room temperature and washed for three times with 0.05% Tween-20 (1× PBS, pH 7.4). Into each well 50 μL of detection antibody dyed-IBV-Ab was added followed by incubation for 1 h at room temperature and washed with 0.05% Tween-20 (1× PBS, pH 7.4) three times. 100 μL of filtered PBS (pH 7.4) was then added into each well and followed by the fluorescence intensity analysis (Ex=488 nm, Em=519 nm) on a microplate reader. All samples were tested in triplicate. Table I summarizes the results of the detection of IBV spiked chicken blood by presented immunosensor and ELISA. A good recoveries and consistency of the spiked IBV are presented. The standard derivations (SD) were 1.0~10 % for both of methods. The total detection time from adding a sample was around 10 min. The results clearly demonstrated that the presented immunosensor is capable of the single-step detection of IBV in chicken blood sample and its high accuracy.

**IV. CONCLUSIONS**

In this study, we reported a proof-of-concept MoS$_2$-based immunosensor on a thread-based microfluidic network for rapid IBV detection. The distinct quenching property of MoS$_2$ on the fluorophore during the antigen-antibody reaction was adopted. The thread-based network interconnected by knots to achieve the fluid mixing and separation. IBV standards and

| Spiked concentration (×10$^6$ EID$_{50}$ per mL) | Immunosensor SD (%) | ELISA SD (%) |
|-----------------------------------------------|---------------------|-------------|
| 1.0                                           | 0.98                | 3.1         |
| 5.0                                           | 4.63                | 9.3         |
| 10.0                                          | 9.80                | 6.2         |
| 50.0                                          | 46.5                | 1.0         |

TABLE I

**COMPARISON OF AVIAN DETECTION USING PRESENTED IMMUNOSENSOR AND ELISA METHOD**
spiked chicken blood sample were successfully detection with high specificity and a detection of limit of 4.6×10² EID₅₀ per mL. The present immunosensor demonstrated a good linearity and validated with ELISA method. In comparison with conventional immunological tests, the presented method have many advantages, such as ease of local manufacture, small consumption of reagents and samples, high sensitive and short time of analysis. All these characteristics allow for the use of this technology for rapid, prompt on-site IBV detection.

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