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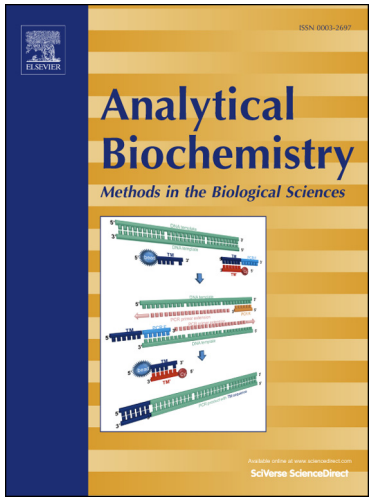

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Rapid and Sensitive Detection of *Maize Chlorotic Mottle Virus* Using Surface-Plasmon-Resonance Based Biosensors

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Subject category: biosensors

Short title:

A novel SPR based biosensor for selective detection of MCMV virus.

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Abstract:

We report a biosensor based on Surface Plasmon resonance (SPR) for the selective detection of *Maize Chlorotic Mottle Virus* (MCMV). 11-mercaptoundecanoic acid was applied on a gold surface to form a self-assembled monolayer and a layer of anti-MCMV antibody was crosslinked on the surface for specific recognition of MCMV. The effects of coupling reaction time and antibody concentration on detection sensitivity were studied. The coverage mass change is a function of the concentration of MCMV with a dynamic range from 1 ppb to 1000 ppb. The detection limit is approximately 1 ppb which is about two orders of magnitude higher than that of the exiting ELISA method. The developed SPR sensor showed highly specific recognition for both purified MCMV and crude extracts from real-world samples.

Key words: *Maize Chlorotic Mottle Virus*, Surface Plasmon Resonance, biosensor, virus detection.

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1. Introduction

Maize chlorotic mottle virus (MCMV) is one type of virus from the genus Machlomovirus and was first found in maize from Peru in 1973 [1, 2]. MCMV is one of the most important plant pathogenic viruses, which can induce various symptoms such as mild mosaic and lead crop losses of 10-15% in natural field infections and losses of up to 59% in experimental maize plots [3]. The combination of MCMV with the maize dwarf mosaic virus (MDMV), sugarcane mosaic virus (SCMV) or wheat streak mosaic virus (WSMV) may also cause a severe reaction known as maize lethal necrosis (MLN) [4-7]. In addition, it can also infect wheat [8], barley [9], sorghum [10] and some weeds [11]. MCMV can be transmitted by infected maize and its vectors of leaf beetles. Due to the potential threat to the production of maize crops, it was listed as a quarantine pest by the Chinese government in 2007. In 2009 and 2011, MCMV was identified in maize seeds imported from the United States [12], Germany [13] and Argentina [14], and now MCMV is considered to have a high risk of introduction into China because of increased international exchanges of maize seeds.

Methods used in the detection of MCMV include Enzyme-Linked ImmunoSorbent Assay (ELISA), reverse transcript polymerase chain reaction (PCR), real time PCR, etc [15-18]. Although these methods can provide the desired sensitivity, specificity, and selectivity, most of these methods require intensive sample preparation, labeling of antigen or antibody, and long analysis time [19]. Under certain circumstances, there is a demand for rapid detection of MCMV, such as at customs for fast scanning of suspected samples. To our best knowledge, no previous reports have demonstrated a method for sensitive detection of MCMV in minutes.

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In this work, we report a biosensor based on surface plasmon resonance (SPR) for the detection of MCMV. SPR is a surface-sensitive optical technique and has been widely used for monitoring interactions of molecules occurring in very close proximity to transducer surfaces [20-22]. Rapid and real-time analysis without labeling [23], low detection limits [24,25], identifying specific analytes in complex matrices without sample preparation,[26] and possible automation and miniaturization [27] make the SPR a very useful tool in analysis of biosamples. In our design, 11-mercaptoundecanoic acid (11-MUA) was applied on a gold surface to form a self-assembled monolayer. Then a layer of anti-MCMV antibody was crosslinked on the surface for specific recognition of MCMV. Several parameters affecting the performance of the immunosensor have been optimized, such as antibody-fixed time and concentration of the antibody. The detection of both purified MCMV virus and crude extracts from real samples with or without MCMV have been studied using the developed SPR sensor. The designed biosensor showed highly promising analytical characteristics, such as high sensitivity, selectivity, and repeatability. The immunosensor may be used for the rapid and reliable determination of MCMV.

2. Materials and methods

2.1. Materials and reagents

Phosphate buffered saline (PBS, pH 7.4), 11-Mercaptoundecanoic acid (MUA), 11-Mercaptoundecanol (MUO), absolute ethyl alcohol, coupling agents, including 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), bovine serum albumin (BSA), and N-hydroxysulfosuccinimide (Sulfo-NHS), were purchased from

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Sigma Aldrich. MDMV, WSMV, SCMV and MCMV ELISA detection kit were purchased from Agdia (Elkhart, USA). MCMV and its monoclonal antibody were obtained from the Institute of Plant Quarantine, Chinese Academy of Inspection and Quarantine (CAIQ). The MCMV viruses were inoculated in corn, and the infected tissues were harvested after 14 days. Purification of MCMV was done according to a reported procedure [28]. The specificity test of the MCMV showed that it has no cross-reaction with the viruses in same family, such as tomato bushy stunt virus (TBSV) and carnation ringspot virus (CRV). The concentration of purified MCMV virus was measured at 260 nm using a UV-Vis spectrophotometer (Thermo Company, Evolution 300).

2.2. SPR sensor

In the study, a SPR sensor (SPR Navi 200, BioNavis, Finland) with a manual sample injection system (12-port valve) and a temperature control of liquids was used. The MCMV recognition was measured in the fixed angle mode of the SPR sensor equipped with a SPR Navi Autosampler. The SPR cell and gold chips were thoroughly washed in deionized water and ethanol with an ultrasonic cleaner. A flow rate of 10 μL min^{-1} was used throughout these experiments and all samples were monitored at a constant temperature of 18 ^{o}C.

2.3. Surface pretreatment

Prior to functionalization, a gold SPR chip was treated with ammonia/hydrogen peroxide, which was composed of 1 part of 30% ammonia (NH_4OH), 1 part of 30% hydrogen peroxide (H_2O_2) and 5 parts of water. After mixing, the solution was heated to

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its boiling temperature, and then cooled to approximately 80-90 ^{o}C. The temperature was adjusted to a value where only slight bubbling of the solution was observed. A gold coated SPR chip was submerged in it for approximately 10 minutes. The chip was then removed from the solution and rinsed with pure water and blow-dried in nitrogen. The freshly prepared chip was used immediately in the next step for surface functionalization to avoid contamination on exposure to 'toxic' vapors in the air, such as trace thiol, NO, NO_2, etc.

2.4. Surface modification

Freshly cleaned gold chips were stored overnight in a 1 mM mixture of ethanolic solution consisting of 11-MUA and 11-MUO. Self assembled monolayers (SAMs) of 11-MUA and 11-MUO were formed on the gold film via Au-SH interaction. The chips were then thoroughly rinsed with distilled water before being immersed in a solution of 0.5 M Sulfo-NHS and 0.2 M EDC in MES for 1 h. This was followed by thorough rinsing of the chips in distilled water to remove excess chemicals that were physically adsorbed on the surface. Then the MCMV antibody was immobilized on the chips via incubating the chips in a solution that contains 0.2 mg/mL of MCMV antibody in a Tris-HCl (0.05 M, pH 8.5) buffer for 3 h. The chips were rinsed again to remove any excess antibody physically adsorbed on the surface. Finally, a 2 mg/ml BSA was used to block the residue NHS-ester sites on the surface via dipping the chips in a solution of BSA for 1 h. The modified chips were stored in a phosphate buffer solution (pH 7.4, 0.1M) at 4 ^{o}C before use.

2.5. MCMV assay

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A chemically modified chip was placed in the holder of the SPR sensor. A working solution (0.1M PBS, pH7.4) was injected into the flow cell through the low pressure injection port sample loop system. The flow rate was maintained at a constant rate of 10 μL/min throughout the experiment. In our experiment, the fixed angle model was used for the detection. 200 μL of MCMV resuspended in working solution of different concentrations was injected through the injection port sample loop system which allowed for the continuous exposure of the chip to the desired solution. The injection of the analyte was done after a stable baseline was obtained. To examine the detection specificity, 1 ppm MDMV, SCMV and WSMV in 0.1M PBS were used as well.

2.6 Infected sample detection assay

A 0.2 mg sample of fresh leaves was simply pulverized with liquid nitrogen for 2 min and the powder was suspended in PBS buffer. After centrifuging at 5000 r/min for 5 min, the supernatant was used for the SPR detection.

2.7 ELISA method for MCMV detection

A direct ELISA method was conducted in our experiment with an ELISA kit purchased from Agdia.

3. Results and discussion

3.1. Optimization of the modification

3.1.1. Effect of the ratio of 11-MUA and 11-MUO on the sensitivity

The ratio of the two thiols can have a major effect on the immobilization capacity of the surface. Five different concentration ratios of 11-MUA and 11-MUO were

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investigated, here the total concentration of these two thiols was 1 mM. Figure 1 shows that the 1:10 ratio of 11-MUA and 11-MUO resulted in the best sensitivity of the sensor.

3.1.2. Effect of coupling reaction time and antibody concentration on the sensitivity

It is expected that the efficiency of carbodiimide coupling reaction will affect the sensing signals. To optimize the conjugation chemistry to obtain the highest sensitivity toward MCMV, the effects of antibody coupling reaction time and the antibody concentration on the sensing signals are investigated and the results are shown in Figure 2, Figure 2a shows that a 3-h antibody coupling time is needed for an optimized SPR response. Figure 2b shows that an antibody concentration above 200 ng/mL is needed for an optimized SPR response.

Based on these observations, the chips used in these experiments were all prepared by immersing the 1 mM mixture of 11-MUA and 11-MUO (concentration ratio was 1:10) modified chips into a 200 ng/mL MCMV antibody for 3 h prior to measurement of MCMV. At this optimized surface modification condition, the SPR sensor has the highest sensitivity for the detection of MCMV with minimum time and smallest amount of antibody.

3.2. Detection of MCMV

The SPR responses to MCMV as a function of time were shown in figure 2a.The concentration of MCMV in the solution was varied from a low concentration of 1 ppb to a high concentration of 1000 ppb. When a MCMV solution was injected after a baseline was established, a SPR response was observed after 1-2 min due to the interaction of

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MCMV with antibodies on the surface, which changed the refractive index of the surface. The 1-2 min delay was the time required for the MCMV samples to travel through the tubing to the Au substrate. It was observed from figure 3a that the SPR responses (In our experiment, the response unit is ng/cm^2, here 0.1 ng/cm^2 equals to 1 RU) increased with higher MCMV concentration. Figure 3b shows the SPR responses to a variety of concentrations of MCMV from 1 ppb to 1000 ppb. The noise of the SPR instrument is 1 pg/mm^2, which equals to 1 RU. According to analytical chemistry terms, a signal/noise ratio of 3 is regarded as the detection limit. Since the SPR response of the sensor was 3.5±0.6 ng/cm^2 on exposure to 1 ppb MCMV injection, we drew the conclusion that the detection limit was approximately 1 ppb.

The antibody modified SPR sensor demonstrated excellent stability for more than 1 month when stored in the buffer solution at 4 ^{o}C as evidenced by the similar sensing response as shown in Figure S1 in the supporting information. We also investigated the chip-to-chip reproducibility with different Au chips. Exposure of a 500 ppb solution of MCMV to three different SPR chips prepared under the same conditions also caused similar SPR response with the standard deviation within 10% indicating an acceptable chip-to-chip reproducibility as shown in the Figure S2 in the supporting information.

Currently, ELISA is the most popular method for the detection of MCMV and identification of infected maize samples. Uyemoto [10] reported a 100 ng/mL detection limit for MCMV by using the ELISA method. For direct comparison, in this work, we also performed ELISA for the detection of MCMV. A variety of concentrations of purified MCMV standards from 1 ppb to 1000 ppb were detected using the direction ELISA method. The qualitative detection results were determined following guidelines set in the work of Beaty et al.[29] The negative control P/N must be < 2.0 and the

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positive control P/N must be ≥2.0 for the test to be valid. Therefore, a positive test result was obtained when the P/N of the test sample was > 2.0. As shown in table S1, we also obtained a detection limit of approximately 100 ppb, which agreed with the result of Uyemoto's reports. Therefore, the sensitivity of our SPR sensor is approximately two orders of magnitude lower than that of the ELISA method for the detection of MCMV. In addition, it takes almost 2 days to perform an ELISA detection. The developed SPR sensor is a rapid detection method with a detection time of approximately 30 minutes.

3.3. Specificity of the SPR sensor

To learn the specificity of the SPR sensor for MCMV, the responses of the sensor to three positive controls of zea mays viruses, MDMV, WSMV, and SCMV at a concentration of 1000 ng/mL were used for comparison. These viruses are often found in mixed infections with *maize chlorotic mottle virus*, which may interfere with the detection of MCMV. The potential interference of the mixed viruses on the SPR sensor was evaluated and results showed that the SPR sensor had none or little response to MDMV, WSMV, and SCMV. As shown in figure 4, the SPR responses were 6.50±0.42 ng/cm^2 and 6.87±0.51 ng/cm^2 upon exposure to 1000 ppb MCMV and 1000 ppb MCMV +1000 ppb MDMV + 1000 ppb WSMV + 1000 ppb SCMV, respectively. It showed that the MDMV, WSMV, and SCMV do not significantly interfere with the detection of MCMV. The high selectivity is due to the high specificity of the antibody to MCMV over other plant viruses.

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3.5. Detection of MCMV crude extraction from real-world samples

To further evaluate the performance of the SPR sensor for MCMV detection, 8 real-world samples (sample 1-8) were tested. As shown in figure 5, strong SPR signals were observed for sample 1-2 and sample 4-7. No response was recorded for sample 3 and sample 8. We also tested these 8 samples using a commercial direct ELISA kit. As stated above, a positive test result was obtained when the P/N of the test sample was > 2.0. The ELISA results in table S1 in the supporting information confirmed the results from the SPR response. Due to the advantage of monitoring the bio-specific interactions from complex fluids, the SPR sensor is capable of identifying specific analytes in complex matrices without sample preparation [30-31]. Therefore, there is no significant interference on the detection of MCMV using crude extraction, and the SPR sensor is a quick and sensitive detection tool for MCMV due to the limited sample handling and preparation. It is further confirmed that the developed SPR sensor is sensitive and reliable for routine testing of MCMV.

4. Conclusion

A novel SPR sensor for MCMV detection has been developed and demonstrated. The detection limit is 1 ppb, which is approximately two orders of magnitude lower than that of the existing ELISA method. The dynamic range of the sensor is from 1 ppb to 1000 ppb. The developed SPR sensor showed high specificity for MCMV compared to the other three typical maize infected viruses, MDMV, WSMV, and SCMV. For a mixture of the above 4 plant viruses, the SPR sensor showed highly specific signal rise

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from MCMV response. The successful detection of crude extraction of infected real-world sample shows the promising application of these sensors in rapid identification of MCMV without cumbersome sample preparation.

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249 GRAPHICAL ABSTRACT (Color picture)

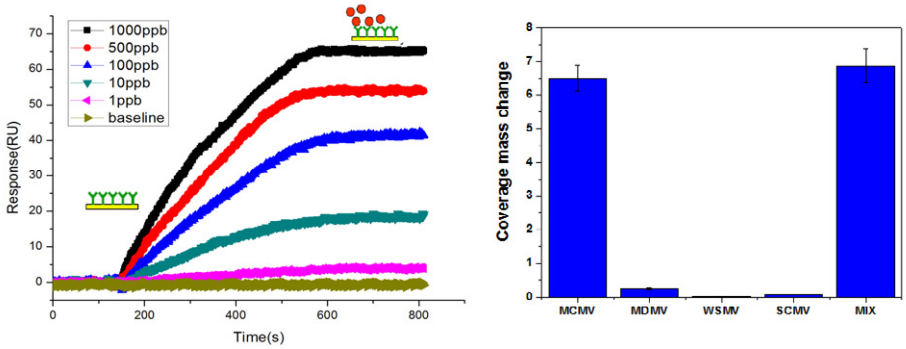

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Figure captions:

Figure 1. Effect of concentration ratio of 11-MUA and 11-MUO on the surface coverage and the subsequent response of SPR sensors to MCMV. The chips were immersed with different ratios of 11-MUA/11-MUO mixtures overnight and then put into a solution of 200 ng/mL antibody in 0.05 M Tris-HCl (pH8.5) for 3h. The SPR responses to MCMV were collected on exposure to 1 ppm MCMV in phosphate buffer (pH 7.4).

Figure 2a. Effect of coupling reaction time on the surface coverage and the subsequent response of SPR sensors to MCMV. The chips were immersed into a solution of 200 ng/mL antibody in 0.05 M Tris-HCl (pH8.5) with different reaction times. The SPR responses to MCMV were collected on exposure to 1 ppm MCMV in phosphate buffer (pH 7.4).

Figure 2b. Effect of antibody concentration on the surface coverage and the subsequent response of SPR sensors to MCMV. The chips were immersed into a solution of antibody with different concentration in 0.05 M Tris-HCl (pH8.5). The SPR response was collected on exposure to 1 ppm MCMV in phosphate buffer (pH7.4).

Figure 3a. SPR response versus time of a SPR chip to MCMV solutions with different concentrations

Figure 3b. SPR response versus concentration of MCMV

Figure 4. Comparison of responses to 1 ppm MCMV and other three viruses (MDMV, WSMV, SCMV) at the same concentration. MIX represents the mixture of MCMV, MDMV, WSMV and SCMV at the concentration of 1ppm.

Figure 5. Comparison of responses to an infected sample and a healthy sample with 1000 ppb MCMV.

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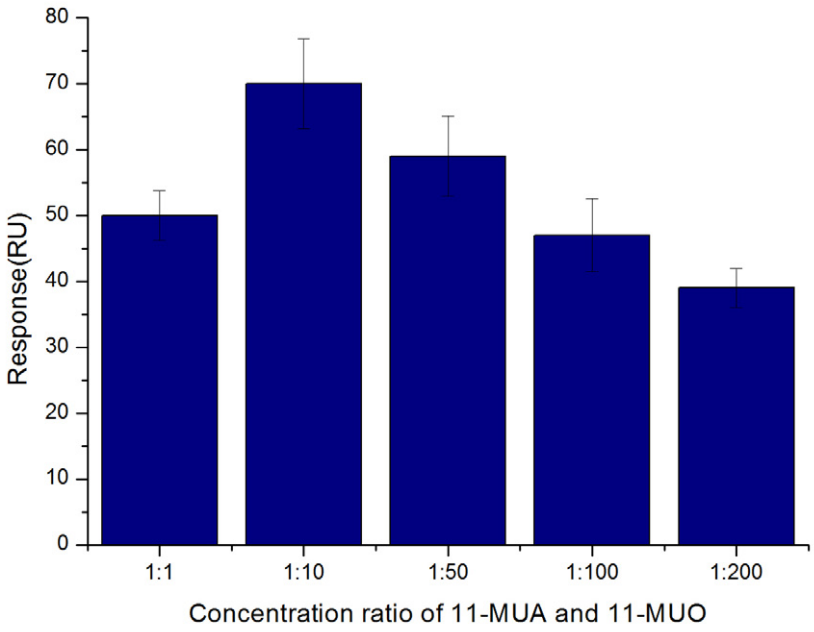

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364 Figure 1

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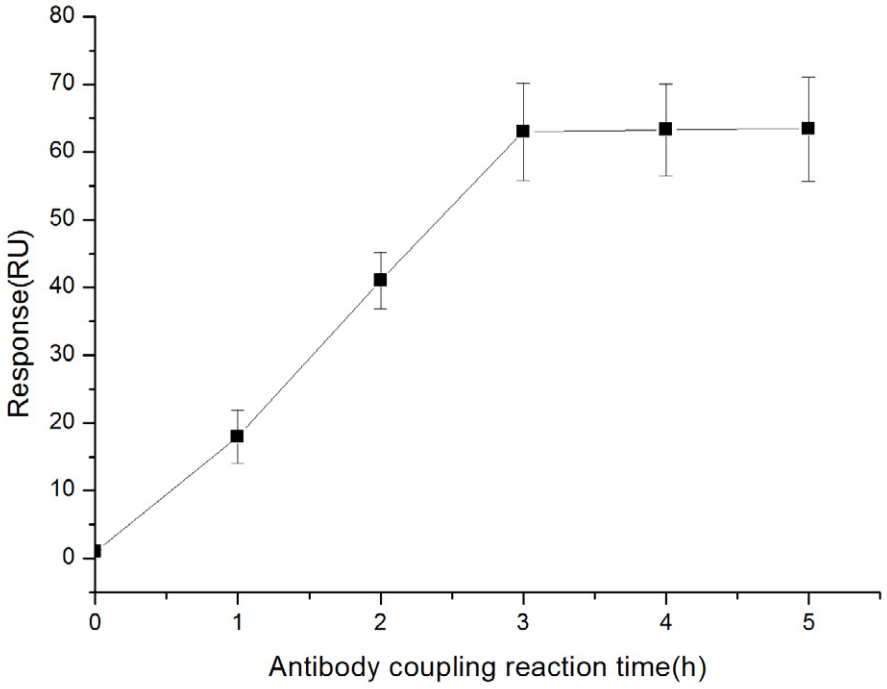

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367 **Figure 2a**

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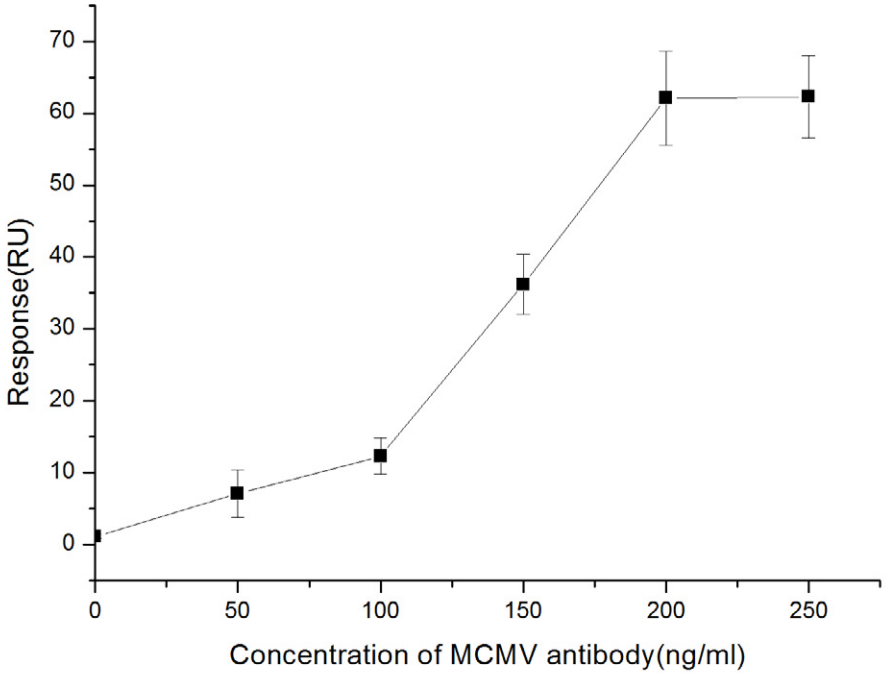

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370 **Figure 2b**

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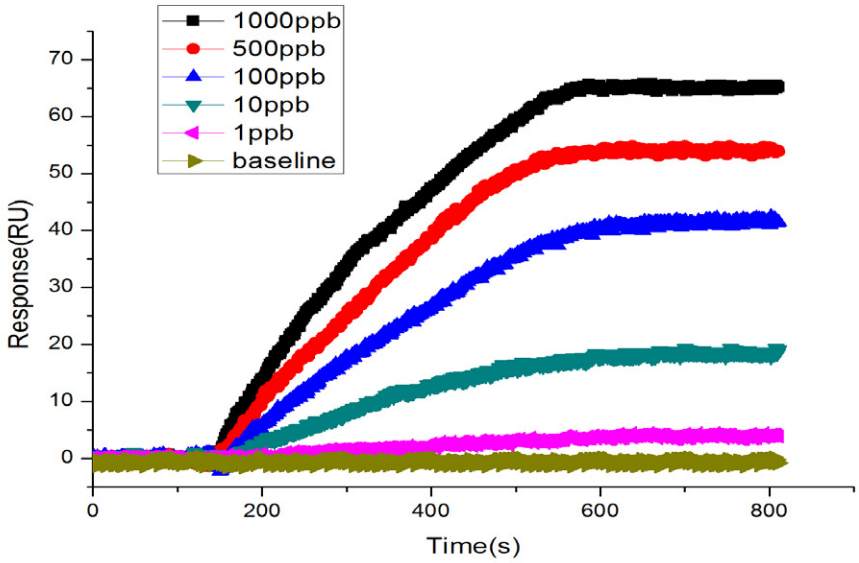

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373 **Figure 3a (Color figure)**

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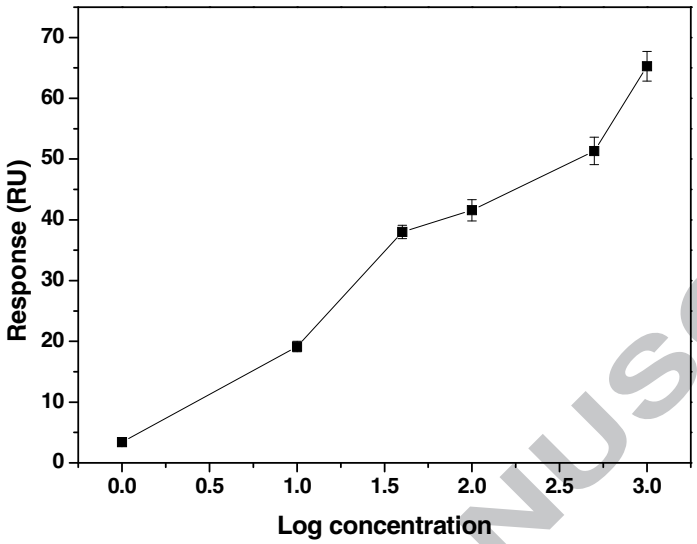

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376 **Figure 3b**

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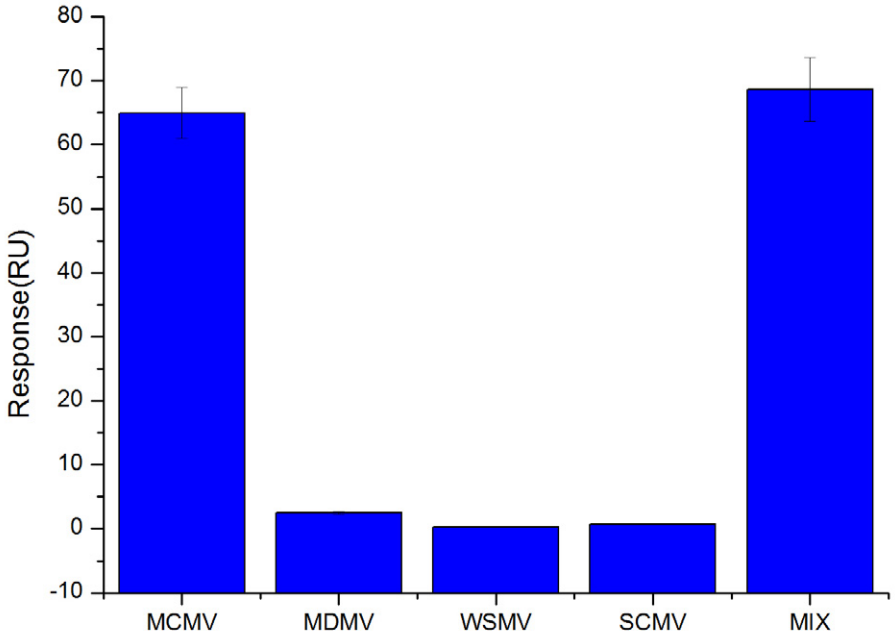

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379 **Figure 4**

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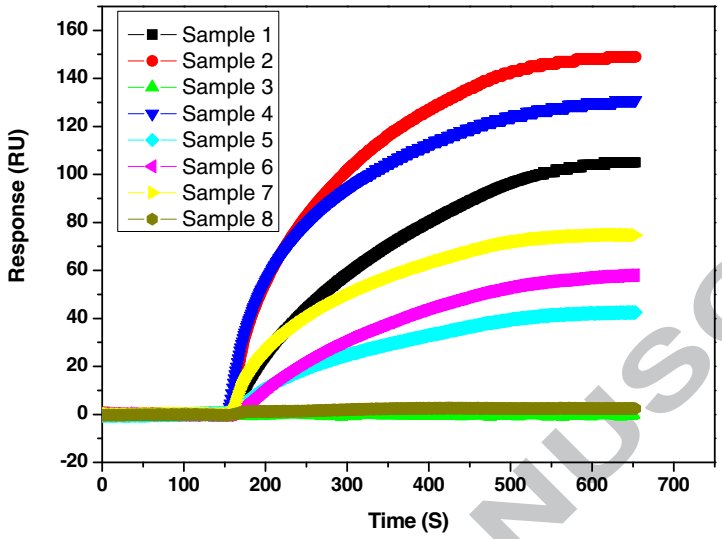
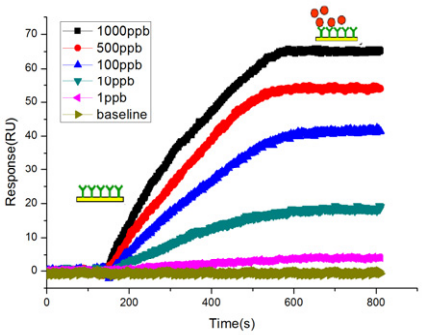


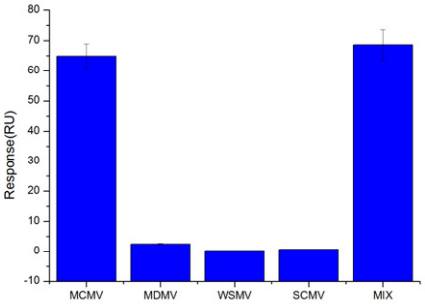
381

382 **Figure 5** (Color figure)

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT





383

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