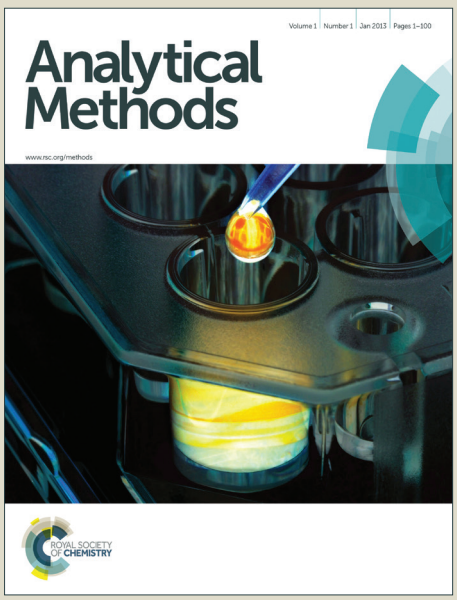
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Quartz crystal microbalance based biosensor for rapid and sensitive detection of *Maize Chlorotic Mottle Virus*

Xin Huang[*,†] *Jianmin Xu,*[†] *Haifeng Ji,*[*,‡] *Guifeng Li*[†], *Hongjun Chen,*[†]

Institute of Plant Quarantine, Chinese Academy of Inspection and Quarantine, Beijing, 100029, People's Republic of China, and Department of Chemistry, Drexel University, Philadelphia, Pennsylvania, 19010, USA

E-mail: huangxin03@tsinghua.org.cn, hj56@drexel.edu

Abstract:

In this work, we report a biosensor based on quartz crystal microbalance (QCM) for the selective and sensitive detection of maize chlorotic mottle virus (MCMV). A mixture of 10:1 3-Mercaptopropanoic acid and 11-Mercaptoundecanoic acid was applied on gold surfaces of QCM crystals to form a self-assembled monolayer, and anti-MCMV antibody was crosslinked on the surface for specific recognition of MCMV. The frequency change of the QCM crystal is a function of the concentration of MCMV with a response range from 250 ng/mL to 10 μg/mL. The standard curve is R^2=0.997, indicating a high reproducibility in MCMV detection. The detection limit is approximately 250 ng/mL, which is similar to that of the existing enzyme-linked immunosorbent assay (ELISA) method. The QCM sensor showed a highly specific and sensitive recognition for both purified MCMV and crude extracts from corn leaf samples.

Keywords: quartz crystal microbalance (QCM), maize chlorotic mottle virus (MCMV).

1. Introduction

Maize chlorotic mottle virus (MCMV) is a single-stranded RNA with a diameter of 30 nm icosahedron [1]. It is a widely distributed plant pathogenic virus that can induce various symptoms such as mild mosaic and lead crop losses in natural field infections[2]. It can spread rapidly via insect vectors and seeds and induce a large area of corn disease such as maize lethal necrosis (MLN) [3-5]. In addition, it can also infect wheat [6], barley [7], sorghum [8], and some weeds [9]. Due to the potential threat to the production of maize crops, it was listed as a quarantine pest. Therefore, rapid detection of MCMV is crucial for the control of MCMV spread.

Current methods for the detection of MCMV include enzyme-linked immunosorbent assay (ELISA) [8, 10, 11], polymerase chain reaction (PCR) [12], real-time PCR [13] etc. These methods provide the desired sensitivity, specificity, and selectivity for MCMV detection; however, they are time-consuming and require intensive sample preparation and labeling of antigens or antibodies [14].

In this work, we report a biosensor based on QCM for the detection of MCMV. QCM is an ultra-sensitive mass sensor. Mass change on a QCM crystal can be measured via the change in frequency of the QCM crystal [15]. Via the piezoelectric effect of a quartz crystal, a QCM sensor measures a change in mass of the crystal, and changes of surface properties, such as viscosity, conductivity, density, dielectric constant, etc[16-19]. Advantages of QCM sensors include simple operation, low cost, fast, label-free, high sensitivity, and capability of real-time applications[20]. QCM have been widely used in the detection of viruses[21] and other chemical and biological species [22].

In our design, a mixture of 10:1 ratio of 3-mecaptoproponic acid [3-MPA] and 11-mercaptoundecanoic acid (11-MUA) was applied on the gold surface of a QCM crystal to form a self-assembled monolayer (SAM). Then anti-MCMV antibody was crosslinked on the surface for specific recognition of MCMV. The detection of both purified MCMV virus and crude extracts from infected plant leaf samples was studied using the developed QCM sensors. The high sensitivity, selectivity, and reproducibility of the biosensors suggest that the QCM immunosensor may be used for the rapid and reliable detection of MCMV.

2. Materials and methods

2.1. Materials and reagents

Phosphate-buffered saline (PBS, pH 7.4, 0.01 M), MUA, 3-MPA, absolute ethyl alcohol, bovine serum albumin (BSA), the coupling agents, including 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS), 2-[morpholino]ethanesulfonic acid (MES) were purchased from Sigma Aldrich (Beijing, China). Maize dwarf mosaic virus (MDMV), wheat streak mosaic virus (WSMV), and sugarcane mosaic virus (SCMV) were purchased from Agdia (Beijing, China). (MDMV, WSMV, SCMV and MCMV ELISA detection kit were also purchased from Agdia. 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 [23]. 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). Healthy and infected tissues, such as infected corn leaves, were also obtained from CAIQ.

2.2. QCM sensor

A QCM sensor (Q-sense E4) with an ismatec reglo digital pump, an electronic unit, and AT-cut QCM crystals (5 MHz) was purchased from Biolin Scientific AB (Finland). The resonance frequency of QCM crystals was 5 MHz and the frequency shift due to mass change was monitored at the third overtone corresponding to 15 MHz. A flow rate of 50 μL min^{-1} was used in these experiments and the results were monitored at a constant temperature of 22 ^{o}C.

2.3. QCM crystal pretreatment

Prior to functionalization, a QCM crystal 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 its boiling temperature, and then cooled to approximately 75 ^{o}C. The temperature was adjusted to a value where only slight bubbling of the solution was observed. A gold-coated QCM crystal was submerged in it for approximately 10 minutes. The chip

was then removed from the solution and rinsed with pure water and ethanol and blow-dried in nitrogen. The freshly prepared QCM crystal was used immediately in the next step for surface functionalization to avoid contamination on exposure to potentially 'toxic' vapors to the surface in the air, such as trace thiols, NO, NO_2, etc.

2.4. Surface modification of the QCM crystals

Surface modification of the QCM crystals was conducted according to a published method[24]. Freshly cleaned gold-coated QCM crystals were stored overnight in a 10 mM ethanolic solution of 11-MUA or a mixture of 3-MPA and 11-MUA (10:1). A self-assembled monolayer (SAM) of 11-MUA or 3-MPA/11-MUA was formed on the gold surface of the QCM crystals via the Au-SH interaction. The crystals were then thoroughly rinsed with ethanol before being immersed in a solution of 0.05 M Sulfo-NHS and 0.2 M EDC in MES for 1 h. This was followed by thorough rinsing of the crystals in ethanol to remove excess chemicals that were physically adsorbed on the surfaces. The MCMV antibodies were immobilized on the crystals via incubating the crystals in a solution that contained 0.2 mg/mL of MCMV antibody in a Tris-HCl (0.05 M, pH6.0) buffer for 3 h at 37 ^{o}C. The crystals were rinsed again to remove any excess antibody physically adsorbed on the surfaces and dried in nitrogen (Figure 1). Finally, the residue NHS-ester sites on the surface were blocked via dipping the crystals in a 2 mg/ml solution of BSA for 1 h. The modified QCM crystals were stored in a phosphate buffer (pH 7.4, 0.1M) at 4 ^{o}C before use.

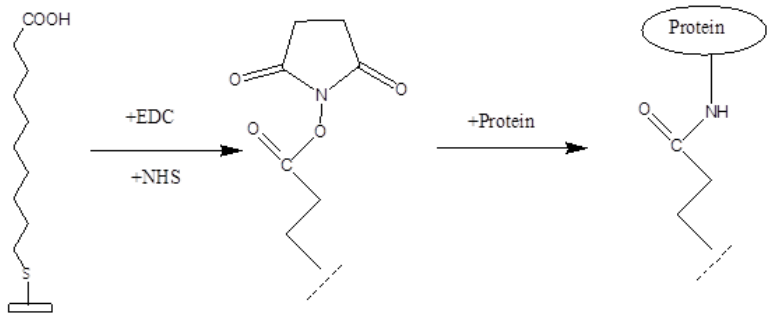


Figure 1. Modification procedure on the gold surface of a QCM crystal.

2.5. MCMV assay

A chemically modified QCM crystal was placed in the QCM-D holder between a pair of O-rings so that the modified side of the crystal was exposed to an analyte sample solution while the other side remained dry. The flow rate was maintained at a constant rate of 50 μL/min at 22 ^{o}C throughout the experiments. A buffer solution (0.1 M PBS, pH 7.4) was injected into the flow cell through a peristaltic pump. The injection of the analyte was done after a stable baseline was obtained, which generally took 10 to 20 minutes. Then MCMV suspended in working solution of different concentrations were injected into the flow cell through the pump which allowed for the continuous exposure of the QCM crystal to the desired solution. To detect specificity of the sensor, 5 μg/mL of MDMV, SCMV, and WSMV diluted in the above PBS were used for comparison.

2.6 Infected sample detection assay

Two separate samples of healthy and infected corn leaves (3 mg each) were transferred into 2 mL centrifuge tubes separately. The tubes were put in a tissue lyser

for 5 min at 1200 rpm. The powders were resuspended in 600mL PBS buffer in the centrifuge tubes. After centrifuging for 5 min at 10000 r/min, the supernatants dissolved in PBS were used for the QCM detection.

2.7 ELISA method for MCMV detection

A direct ELISA method was conducted in our experiment with an ELISA kit purchased from Agdia to demonstrate antigen-antibody activity. The specificity and sensitivity of the ELISA for MCMV was also used for comparison with our QCM sensors. Table 1 shows the optical density (OD) values for MCMV antigen (1 μg/mL), blank control, healthy corn leaves extract (extract negative), and MDMV virus (negative control), each repeated three times. The results confirmed the activity and selectivity of the antibody. A sensitivity of the ELISA for MCMV is 100 ng/mL of MCM [24].

samples	OD value			
	1	2	3	average
MCMV（1 μg/mL）	2.18	2.21	2.15	2.180
Blank control	0.08	0.09	0.09	0.088
healthy corn leaves extract	0.08	0.08	0.09	0.083
MDMV	0.08	0.08	0.09	0.083

Table 1 OD values of TAS-ELISA assay

3. Results and Discussions

3.1. Optimization of the modification

3.1.1. Effect of the ratio of 3-MPS and 11-MUA on the sensitivity

In preparing biosensors, it has been found that SAMs from a mixture of two thiols, typically a shorter one and a longer one, provided a surface for enhanced sensitivity than that from a single thiol compound [25, 26]. This is due to effects of gap filling, higher stability, energy releasing, etc. The sensors prepared from three different concentration ratios of 3-MPS and 11-MUA, 10:1, 5:1, 1:1, and 11-MUA alone were investigated. The total concentration of thiols was 10 mM in each of these solutions.

When an anti-MCMV modified QCM crystal was exposed to a 5 μg/mL MCMW, the frequencies of the crystals decreased due to the adsorption of MCMW on the crystal surfaces (Figure 2). Each test was run on a separate modified QCM crystal. The change in frequency (ΔF) of the modified QCM crystal on exposure to the MCMV was used to evaluate the performance of the crystals since larger ΔF generally gives rise to higher sensitivity of QCM sensors. Figure 3 shows that the crystals prepared from 3-MPA/11-MUA mixture resulted in a similar ΔF of the sensors, and all of them showed a larger ΔF than the sensor from 11-MUA alone. The sensors from 3-MPA/11-MUA mixture at 10:1 ratio showed the fastest response to MCMV. It is noteworthy that the biosensors prepared from 3-MPA alone are not reproducible since a monolayer of 3-MPA is not stable. Thus, in this work, we used the monolayer

prepared from 3-MPA/11-MUA mixture at 10:1 ratio throughout the experiments.

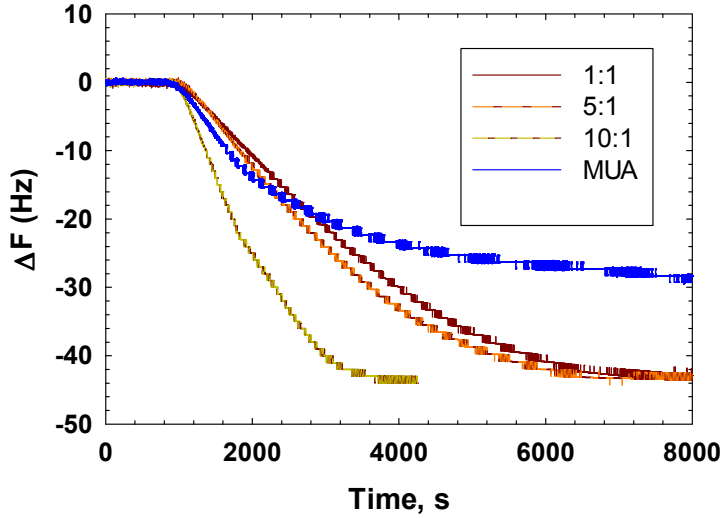


Figure 2. Effect of molar ratio of 3-MPA and 11-MUA (total of 10 mM) on the sensing responses of the QCM sensors to MCMV. In these experiments, QCM crystals were immersed with different ratios of 3-MPA and 11-MUA mixtures overnight and subsequently modified with the MCMV antibody according to the procedure in the preparation section. The ΔF to MCMV was collected on exposure to a 5 μg/mL MCMV solution in phosphate buffer (pH 7.4).

3.1.2. Effect of EDC and NHS concentrations on the sensitivity

It had been reported that the efficiency of carbodiimide coupling reaction will affect the sensing performance of biosensors [27, 28]. The potential effects of EDC/NHS ratio, EDC and NHS concentrations on the sensing performance of the QCM sensors were investigated in this work. Our results suggest that the sensing performance of the QCM sensors is insensitive to the ratios and concentrations of EDC and NHS in a wide range. As one example, in the studied concentration range in Figure 3, the ΔF of the QCM sensors to 5 μg/mL of MCMV are intrinsically the same.

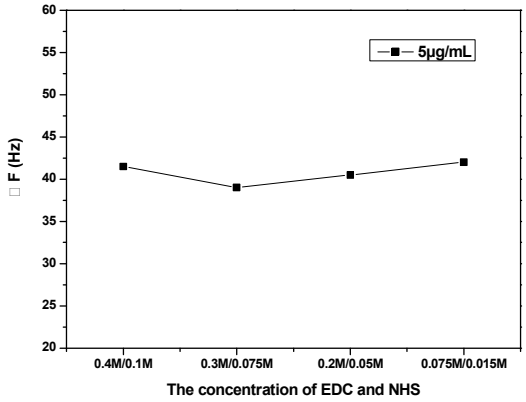


Figure 3. Effect of the concentration of EDC on the sensing response (ΔF) of QCM sensors to MCMV. The QCM responses to MCMV were collected on exposure to 5 μg/mL MCMV in phosphate buffer (pH 7.4). The EDC concentrations were from 0.4 to 0.075 M at a 4:1 ratio of EDC/NHS, followed with MCMV antibody.

3.2. Sensitivity and reproducibility of the QCM sensors for MCMV Detection.

The ΔF responses of the QCM sensors to MCMV as a function of time are shown in Figure 4a (the responses to 10 μg/mL concentration were not included for clearance). The concentration of MCMV in the solution was varied from 250 ng/mL to 10 μg/mL. Figure 4b shows the ΔF as a function of a variety of concentrations of MCMV. The frequency of the crystals decreased with higher MCMV concentration due to more MCMV adsorption on the surface. The noise of the QCM instrument is less than 0.5Hz. According to analytical chemistry terms, a signal/noise ratio of 3 is regarded as the detection limit. Because the frequency shift of the sensor was 1.7 ± 0.2 ng/mL on exposure to 250 ng/mL MCMV, we drew the conclusion that the detection limit was approximately 250 nm/mL. It is noteworthy that the sensitivity of

QCM crystals starting with 11-MUA alone is 1 μg/mL (data not shown), which is consistent with our previous conclusion that the QCM crystals modified with the mixture of 3-MPA and 11-MUA is more sensitive than those prepared from 11-MUA alone for MCMV detection.

Currently, ELISA is the most popular method for the detection of MCMV and identification of infected maize samples. The sensitivity of the ELISA method for MCMV is 100 ng/mL. Therefore, the sensitivity of our QCM sensor is similar to that of the ELISA method for the detection of MCMV. However, the advantage of the QCM method is a detection time of less than 2 hours, as compared to close 2 days for ELISA.

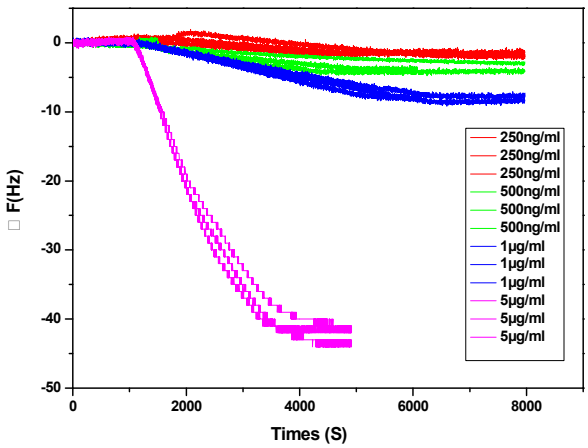


Figure 4a. ΔF of the QCM sensor modified with anti-MCMV crosslinked on 10:1 3-MPA/11-MUA as a function of time on exposure to MCMV solutions.

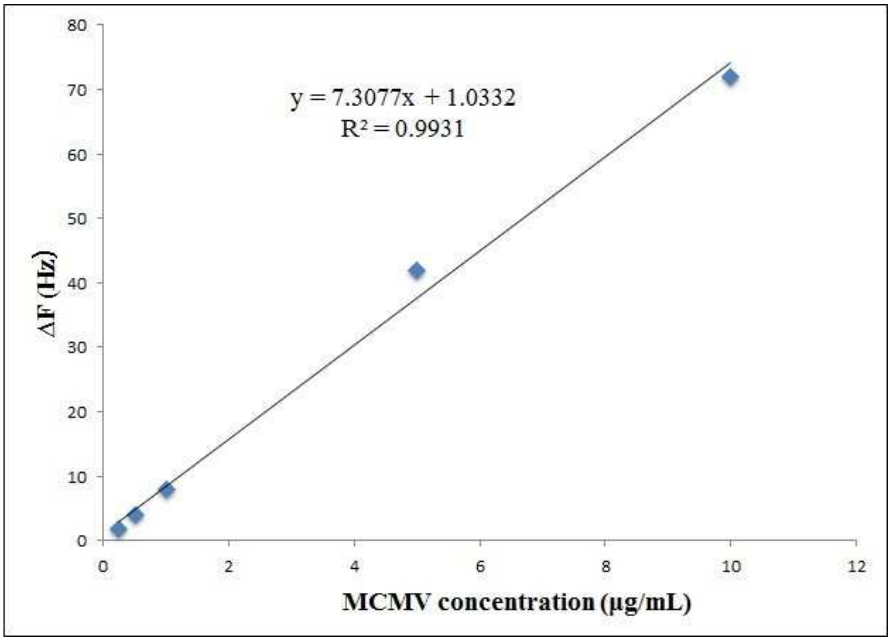


Figure 4b. Standard curve of QCM crystals modified with anti-MCMV crosslinked on 10:1 3-MPA/11-MUA as a function of MCMV concentration.

We could not evaluate the sample-to-sample reproducibility since the binding of antibody-MCMV is irreversible, i.e. once MCMV is bound on the surface, ΔF of the crystal does not change after the crystal is washed with buffer. However, we were able to investigate the sensor-to-sensor reproducibility with different QCM crystals. 15 QCM sensors were prepared under the same conditions and their responses to 5 samples of MCMV solutions at different concentrations were investigated (Figure 4). Each concentration was tested three times with three separate sensors. Exposure of a 250 ng/mL solution of MCMV to three different QCM sensors caused a similar ΔF with the standard deviation within 12%. The same reproducible results were observed for the responses to other concentrations and the standard derivation (SD) and the relative standard derivation (RSD) are show in Table 2. The standard derivation curve

showed a correlation coefficient of R^2=0.9931 and the resulting coefficient of variation (CV%) were less than 10% of the mean averages, respectively, indicating good reproducibility of the QCM sensors for MCMV.

Table 2. Reproducibility of QCM sensors modified with anti-MCMV crosslinked on 3-MPS/11-MUA

[MCMV]	ΔF（Hz）			Average		
(μg/mL）	ΔF1	ΔF2	ΔF3		SD	RSD
0.25	1.9	1.7	1.6	1.73	0.153	8.8%
0.5	3.9	3.6	4.1	3.87	0.33	8.5%
1	7.6	8.4	8	8	0.4	5%
5	41.5	41	43.5	42	1.32	3.1%
10	72	71.5	72.5	72	0.5	0.8%

The antibody-modified QCM sensors demonstrated excellent stability for 1 week when stored in the buffer solution at 4 ℃as evidenced by the similar sensing response as shown in Figure 5. The ΔF change decreased when stored in the buffer for more than 1 week.

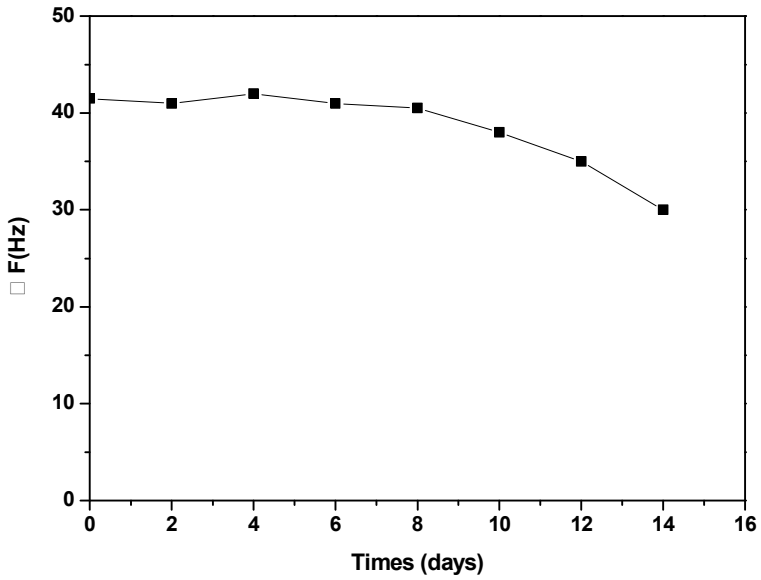


Figure 5. Responses of QCM sensors as a function of store time on exposure to 5 μg/mL of MCMV.

3.3. Specificity of QCM sensor

MDMV, WSMV, and SCMV are often found in mixed infections in *Zea mays viruses* with MCMV and they may interfere with the detection of MCMV. To understand the specificity of the QCM sensors for MCMV, the ΔF of QCM sensors to these positive controls and a mix of four viruses at a concentration of 1 μg/mL were studied for comparison.

Figure 6 shows that the ΔF of QCM sensors had little or no response to MDMV, WSMV, and SCMV. The high selectivity to MCMV is due to the high specificity of the antibody to MCMV over other plant viruses.

In another comparison in Figure 6, the ΔF of the QCM sensors were -40 Hz and -46 Hz on exposure to 5 μg/mL MCMV alone and 5 μg/mL MCMV + 5 μg/mL MDMV + 5 μg/mL WSMV + 5 μg/mL SCMV, respectively. It also suggests that the

MDMV, WSMV, and SCMV do not significantly interfere with the detection of MCMV.

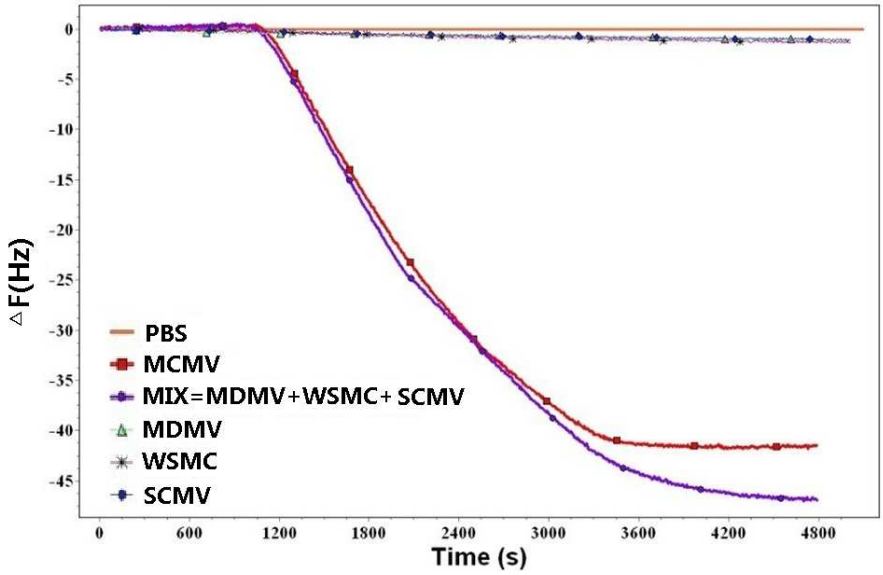


Figure 6. Comparison of responses of the QCM crystals to 5 μg/mL MCMV and the other three viruses— MDMV, WSMV, and SCMV—at the same concentration. "MIX" represents the mixture of MCMV, MDMV, WSMV, and SCMV at the concentration of 5 μg/mL each.

3.4. Detection of MCMV in crude extractions from plant samples

To evaluate the feasibility of the QCM sensors for samples from plants, both infected and healthy corn leaves were tested. In our experiments, a 0.2 mg sample of healthy or the MCMV infected maize leaves were transferred into 2 mL a centrifuge tube. Then the tube was put in a high throughput tissue lyser for a few minutes and the resulted powder was dispersed in a PBS buffer. After centrifuging at 10000 r/min for 5 min, the supernatant was diluted with PBS at 1:19, 1:9, 1:4, 1:1 Ratios and the

diluted solution was used for the QCM detection. Figure 7 shows that a significant change in the frequency of the QCM sensors to the infected maize sample, and no response was observed to the healthy sample. The results suggest that the QCM sensor is capable of identifying MCMV in complex matrices with crude sample preparation.

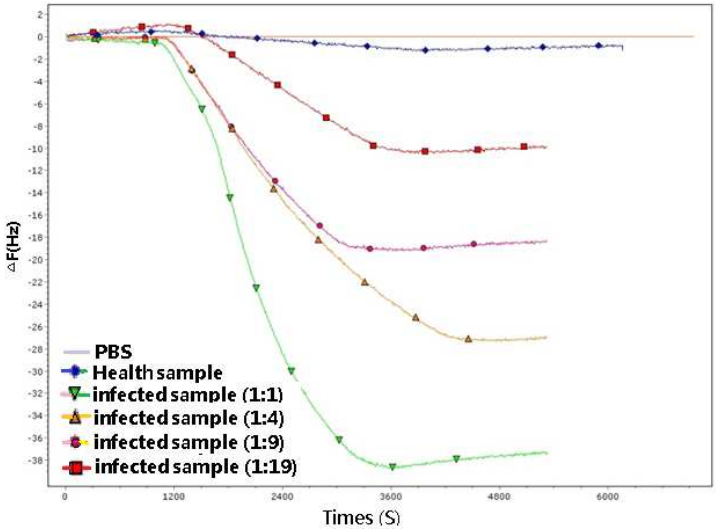


Figure 7. Comparison of ΔF of QCM sensors to an infected sample at different concentrations and a healthy sample.

3.5. Dissipation change on MCMV absorption on the QCM.

Recently, a QCM-dissipation or QCM-D instrument was developed. It can measure ΔF and the change in the energy dissipation factor, ΔD, simultaneously. The ΔD provides information on the viscoelastic properties of the adsorbed species. [29-30]. Figure 8 shows that when the MCMV concentration was less than 1 μg/mL, small absorption of MCMV on the QCM surface caused relatively great dissipation. This suggests MCMV is loosely attached on the surface. When the MCMV concentration is greater 10 mg/mL, the slop of the ΔD/ΔF decreased, indicating the MCMV film on the surface grew quicker and more compact. This trend is further confirmed that,

when ΔF is greater than 50, the ΔD reached to a plateau, suggesting the MCMV film on the surface is compact and rigid.

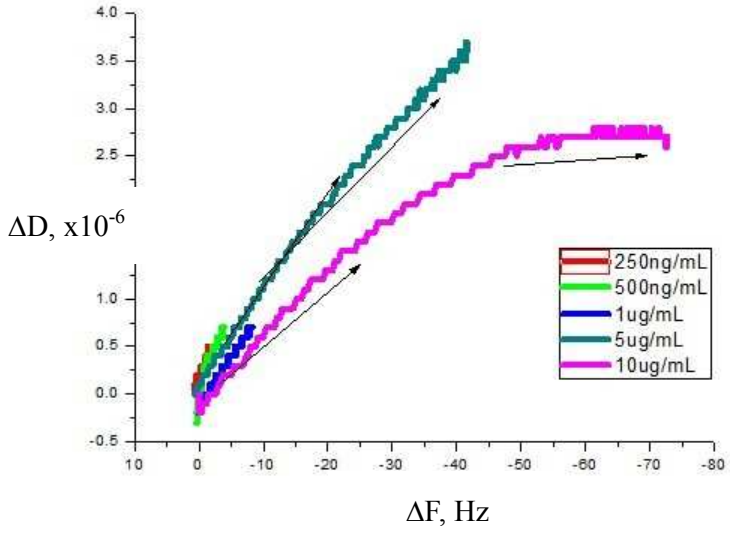


Figure 8. Dissipation change vs. ΔF when the QCM plates were exposed to MCMV solutions with different concentrations.

4. Conclusion

A QCM sensor for MCMV is reported. The detection limit is 250 ng/mL, which is similar to that of the existing ELISA method. The response range of the sensor is from 250 ng/mL to 10 μg/mL. The QCM sensor showed high sensitivity and specificity for MCMV. The sensor demonstrated promising applications in efficient and rapid identification of MCMV in crude extractions of infected plant leaves without cumbersome sample preparation.

Acknowledgment We thank the important National Science and Technology specific projects (2013ZX08012-001).

Reference

1. S. Lommel, T. Kendall, N. Siu and R. Nutter, *Phytopathology*, 1991, 81, 819-823.
2. E. M. Larsson, M. E. Edvardsson, C. Langhammer, I. Zoric and B. Kasemo, *Review of Scientific Instruments*, 2009, 80, 125105-125105-125110.
3. X. H. Zhang, *Physical Chemistry Chemical Physics*, 2008, 10, 6842-6848.
4. B. Wu, C. Li, H. Yang, G. Liu and G. Zhang, *The Journal of Physical Chemistry B*, 2012, 116, 3106-3114.
5. J. Song, J. Liang, X. Liu, W. E. Krause, J. P. Hinestroza and O. J. Rojas, *Thin solid films*, 2009, 517, 4348-4354.
6. D. Bockelman, L. Claflin and J. Uyemoto, *Plant disease*, 1982, 66, 216-218.
7. J. Castillo and T. Hebert, *Fitopatologia*, 1974, 9, 79-84.
8. J. Uyemoto, *Phytopathology*, 1980, 70, 290-292.
9. L. Nault, D. Gordon, V. Damsteegt and H. Iltis, *Plant disease*, 1982, 66, 61-62.
10. L. Xie, J. Zhang, Q. Wang, C. Meng, J. Hong and X. Zhou, *Journal of Phytopathology*, 2011, 159, 191-193.
11. J.-x. WU, Q. WANG, H. LIU, Y.-j. QIAN, Y. XIE and X.-p. ZHOU, *Journal of Zhejiang University SCIENCE B*, 2013, 14, 555-562.
12. G. HaiYan, Z. YongJiang, Z. ZhiYu, C. HongJun, G. BiDa and Z. ShuiFang, *Acta Phytopathologica Sinica*, 2010, 40, 426-429.
13. Y. Zhang, W. Zhao, M. Li, H. Chen, S. Zhu and Z. Fan, *Journal of virological methods*, 2011, 171, 292-294.
14. K. V. Gobi, K. Matsumoto, K. Toko, H. Ikezaki and N. Miura, *Analytical and bioanalytical chemistry*, 2007, 387, 2727-2735.
15. G. Sauerbrey, *Z Phys*, 1959, 155, 206-222.
16. S. J. Martin, J. J. Spates, K. O. Wessendorf, T. W. Schneider and R. J. Huber, *Analytical chemistry*, 1997, 69, 2050-2054.
17. C. Reed, K. K. Kanazawa and J. Kaufman, *Journal of Applied Physics*, 1990, 68, 1993-2001.
18. Z. Shana, D. Radtke, U. Kelkar, F. Josse and D. Haworth, *Analytica chimica acta*, 1990, 231, 317-320.
19. K. K. Kanazawa and J. G. Gordon, *Analytical chemistry*, 1985, 57, 1770-1771.
20. Y. Montagut, J. G. Narbon, Y. Jiménez, C. March, Á. Montoya and A. Arnau, in *Biosensors - Emerging Materials and Applications*, ed. P. A. Serra, InTech, 2011.
21. C. Mao, A. Liu and B. Cao, *Angewandte Chemie International Edition*, 2009, 48, 6790-6810.
22. D. R. Kauffman and A. Star, *Chemical Society Reviews*, 2008, 37, 1197-1206.
23. C. Niblett and A. Paulsen, *Phytopathology*, 1975, 65, 1157-1160.
24. C. Zeng, X. Huang, J. Xu, G. Li, J. Ma, H.-F. Ji, S. Zhu and H. Chen, *Analytical biochemistry*, 2013, 440, 18-22.
25. N. Patel, M. C. Davies, M. Hartshorne, R. J. Heaton, C. J. Roberts, S. J. Tendler and P. M. Williams, *Langmuir*, 1997, 13, 6485-6490.
26. N. Patel, M. Davies, R. Heaton, C. Roberts, S. Tendler and P. Williams, *Applied Physics A: Materials Science & Processing*, 1998, 66, S569-S574.
27. H.-F. Ji, H. Gao, K. R. Buchapudi, X. Yang, X. Xu and M. K. Schulte, *Analyst*, 2008, 133, 434-443.
28. S. Velanki and H.-F. Ji, *Measurement Science and Technology*, 2006, 17, 2964.
29. C. Larsson, M. Rodahl, F. Hook, *Analalytical Chemistry*, 2003, 75, 5080–5087.
30. S. M. Notley, M. Eriksson, L. Wågberg, Journal of colloid and interface science, 2005, 292, 29-37.