Simple biosensing method to detect DMMP based on QCM transducer and acetylcholine esterase sensitive film

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A simple and stable method to detect dimethyl methylphosphonate (DMMP) was studied. Through direct monitoring of acetylcholinesterase (AChE) inhibition without histological substrate, the concentration of DMMP was sensitively detected. In the experimental setup, a liquid–gas interface was developed to improve the oscillation stability of quartz crystal microbalances (QCMs) and to prevent protein denaturation of AChE. AChE was immobilised on the surface of gold electrodes on QCM by creating a cross-linked gel with glutaraldehyde. Experimental results showed that the prepared biosensor had a frequency fall of ~4 Hz when a concentration of 1 ng/ml increment occurred for liquid DMMP. Furthermore, the detection was repeatable and selective to only DMMP. Frequency response to DMMP in gas phase was also studied. The considerable frequency response and its complete recovery showed that the sensor had wide application potential in attack defence and public safety construction.

1. Introduction: Terrorist attacks by using chemical and biological warfare agents (CBWAs) often occurred in the past decades. Nerve agents such as O-isopropyl methylphosphonofluoridate (sarin) are recognised as the most toxic kind of typical CBWAs and are a real threat to the world [1, 2]. Thus, detection of trace levels of sarin with portable instrument is important. However, due to its high toxicity, a simulant of sarin is indispensible for security reason. Dimethyl methylphosphonate (DMMP) is a good candidate for its low toxicity and similar chemical structure with sarin. Therefore, DMMP has been widely used to replace sarin in laboratory. Development of a rapid, sensitive, and low-cost DMMP sensor will provide good references for establishing effective methods of sarin detection.

Recently, DMMP sensors based on quartz crystal microbalance (QCM) technique have been extensively studied [3, 4]. This detection method has advantages including digital frequency output and fast response. Besides, detection instruments based on this method are of low cost and small scale as well. For DMMP sensors, they should have stable frequency response, high sensitivity to concentration, and good selectivity on DMMP kind of chemical compounds. Among all the design considerations, sensing material is an essential factor that greatly influences the above sensing performance. In this literature, strong hydrogen-bond acidic chemical polymers including poly (vinylidene fluoride) [5], fluoroalcoholpolysiloxane, and fluoroalcoholic linear polysiloxane [6, 7] are mostly applied to detect DMMP. In the above-mentioned polymers, hexafluoroisopropanol is the common functional group [8]. However, in the polymer synthesis process, highly toxic hexafluoroacetone gas is inevitably used and high-pressure reactor is applied, which will impose dangerous workplace to relevant personnel [8–10].

Besides chemical polymers, biomolecules such as antibodies, antigens, and enzymes are excellent substitutes for toxic agent detection. Compared with chemical sensing materials, biomaterials have lower toxicity, higher sensitivity, and specific selectivity [11]. For example, the limit of detection (LOD) of mass sensitive organophosphorus (OP) pesticides biosensors based on acetylcholinesterase (AChE) receptor are as low as -10^{-11} M [12, 13]. In detection, AChE combines with OP compounds through covalent bond, and its enzymatic activity is inhibited. To monitor this inhibition, a histological substrate, 3-indolyl acetate, should always be used to observe the insoluble product catalysed by AChE, indigo pigment by a frequency counter [14]. This complex detection method makes the manipulation of experiment difficult. Moreover, to keep the enzymatic activity, detection experiments are carried out in liquids, whose large damping significantly reduces the working stability of QCMs.

In this Letter, a simple and stable method to detect DMMP based on AChE biosensing material is developed, as illustrated in Fig. 1. Without histological substrate, the inhibition and reactivation of AChE on one face of QCM can be directly monitored, and the concentration of DMMP can be determined further. To improve the stability of the biosensor, a gas–liquid interface was built in the experimental setup. With this method, the sensitivity, repeatability, and selectivity of the biosensor for detection of DMMP in both liquid and gas phase are obtained.

2. Experimental results

2.1. Reagents: AChE (EC 3.1.1.7; type VI-S) from electric eel was purchased from Sigma-Aldrich (MO, USA). It is a solid biomaterial whose activity is 250 UN per milligram (U/mg), and should be stored at −20°C before used. Bovine serum albumin (BSA, 98 wt.% purity) and glutaraldehyde (50 wt.% solution in H_2O) were obtained from J&K Scientific Corporation (Beijing, CN) and used as received. Phosphate buffered saline (PBS) was bought from Pierce (IL, USA). It is an ideal concentrated buffer, and should be diluted 20-fold in water for use in cross-linking. When diluted, the solution yields 10 mM sodium (Na) phosphate, 0.15 M Na chloride (NaCl), pH 7.5. The detection target, DMMP (98 wt.% of purity), was purchased from Sun Chemical Technology Company Limited. (Shanghai, CN) and was used as received. All other chemicals were of analytical reagent grade.

2.2. System set-up: AT-cut QCMs with their fundamental frequency of 10 MHz (JSL45, Wuhan Hitrusty Electronics Company Limited, CN) were used as the transducer. Gold electrodes of 5 mm diameter were covered on both sides of QCMs to make a reaction area and a connection with the oscillator and frequency counter. The construction of the

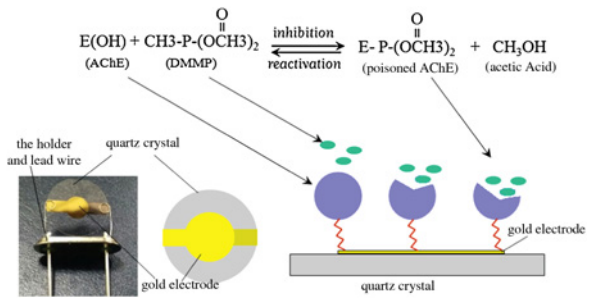

Fig. 1 *QCM biosensor and its principle for the detection of DMMP*
a Photograph of QCM biosensor
b Top view of the QCM biosensor
c Cross-section of the QCM biosensor and the chemical reaction

detection system was depicted in Fig. 2. QCMs were mounted inside a gas room with a plug holder. Excited by an oscillator (QCM-5, Shenyang Vacuum Technology Institute, CN), the frequency shift of the QCM was monitored by a frequency counter (SS7200, Shijiazhuang Suin Digital Instruments Company Limited, CN). The frequency counter was connected to a computer system via a general-purpose interface bus (GPIB) interface board, and the results were visualised by a corresponding software for analysis. The gas room was designed to have both an inlet and an outlet for gases flowing in and out, respectively.

During the detection, QCMs were in dynamic N_2 or DMMP mixture with N_2 at a flow rate of a 1000 ml/min. The dynamic gases were generated by a dynamic generator (MF-3C, China National Metrology Technology Development Corporation, CN). The tests were carried out at room temperature (25°C) and 75% humidity. Steady humidity was controlled by the saturated NaCl solution at a fixed temperature to ensure the stability and accuracy of the test results. In this way, a gas–liquid interface was generated. On the one hand, the frequency measurement was implemented in gas phase so that the oscillation stability of QCMs will be greatly improved. On the other hand, AChE molecules were always in a wet environment during the test experiment, thereby preventing protein denaturation.

2.3. Enzyme immobilisation: Cross-linking method was utilised to immobilise AChE on the surface of QCM [12, 14, 15]. First, the QCM was prepared step by step as follows: soaked in 1.2 M Na hydroxide for 5 min, washed with distilled water, immersed in 1.2 M HCl for 5 min, washed with acetone and distilled water, and finally dried in a vacuum oven for 30 min at 60°C. Second, three samples including 10 µl of 250 U/ml AChE in PBS, 5 µl of 5 wt.% BSA in PBS, and 5 µl of 5 wt.% glutaraldehyde in distilled water were mixed together in a centrifuge tube by a vortex mixer for uniformity. Third, a small amount (2 µl) of the mixed samples was dropped onto the surface of the QCM, and then incubated for −10 h (overnight) at 4°C. Finally, the modified

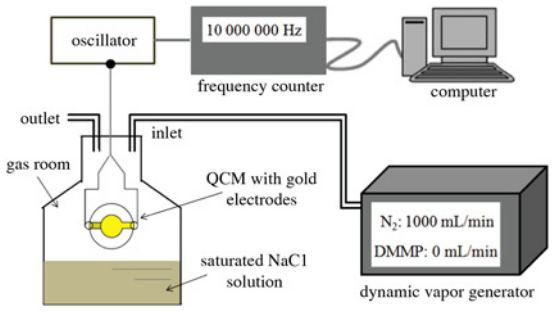

Fig. 2 *Experimental setup of the biosensor*

QCM was rinsed with 1 wt.% glycine in PBS and distilled water successively several times and dried in vacuum for 1.5 h at 30°C. To prevent protein denaturation, the QCM was kept in PBS solution at 4°C before use.

3. Results and discussion

3.1. Adsorption of AChE on electrodes of QCMs: As described in Section 2, in the experiment, 2 µl of immobilisation gel containing 0.25 U, i.e. 1 µg AChE, was dropped onto each electrode of QCMs. Sauerbrey [16] presented the frequency shift expression of AT-cut QCMs, as follows:

$$\Delta f = -\frac{2f_0^2 \Delta m}{A\sqrt{\rho_q \mu_q}} \tag{1}$$

where f_0 is the fundamental resonant frequency of QCM, ~10 MHz in this work; A denotes the surface area of the electrode, about 19.625 mm^2 (5 mm in diameter of the electrode); the density ρ_q and shear modulus μ_q of AT-cut quartz are 2.648 g/cm^2 and $2.947 \times 10^{11} g/(cm\,s^2)$, respectively. In our experiments, if we suppose that all AChE molecules are immobilised successfully on the electrodes (the addition of mass on the electrode $\Delta m = 1$ µg), frequency changes Δf could be quantitatively predicted as −1151 Hz.

Fig. 3 shows the immobilisation results of AChE on electrodes of 30 different QCMs. As shown in Fig. 3*a*, the average frequency of 20 QCMs after AChE immobilisation f_1 in N_2 is 9.9835891 MHz. Compared with that of fundamental frequency f_0 of clean QCMs, 9.985162.6 MHz, the difference is −1573.5 Hz, which is smaller than the predicted result, −1151 Hz. The extra frequency shift of

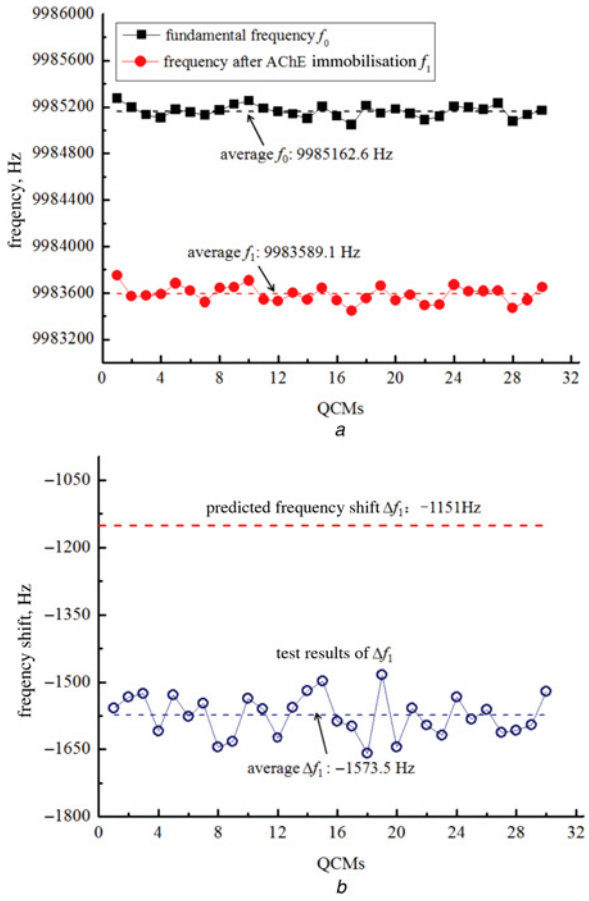

Fig. 3 *Immobilisation results of AChE on electrodes of QCMs*
a Fundamental frequency and frequency after immobilisation of 30 samples
b Consistency and stability of the immobilisation of AChE

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−422.5 Hz can be contributed to the mass of the cross-linker. We believe that AChE has good adsorption on electrodes of QCMs.

Although different from each other, all frequency shift values are smaller than the predicted one, as clearly shown in Fig. 3*b*. Using the following equation [17]:

$$\mathrm{SDR} = \frac{\mathrm{SD}(\Delta f)}{\mathrm{AM}(\Delta f)} \tag{2}$$

standard deviation ratio (SDR) of frequency shifts Δf can be calculated to be 2.88%. SD(Δf) and AM(Δf) are the SD and average of frequency shifts, respectively. This SDR value is low enough to show reproducibility of the immobilisation of AChE, considering the normally accepted 5% level for a reasonable analytical method [13]. The tiny fluctuation of frequency shift is due to the different amount of immobilisation gels dropped onto different electrodes for the error of pipette used in this experiment.

3.2. Liquid DMMP detection results: To validate the frequency response of DMMP, QCMs_{1-7} in Fig. 3 were immersed in DMMP solution for 5 min at concentrations ranging from 0 (distilled water) to 150 ng/ml, in increments of 25 ng/ml. Afterwards, all seven QCMs were dried in a vacuum for 1.5 h at 30°C. Then, their resonant frequencies are monitored. As indicated in Fig. 4*a*, when the concentration of DMMP varies from 0 to 100 ng/ml, the frequency shifts decreases until −401 Hz gradually, thereby indicating an adsorption characteristic of DMMP to AChE.

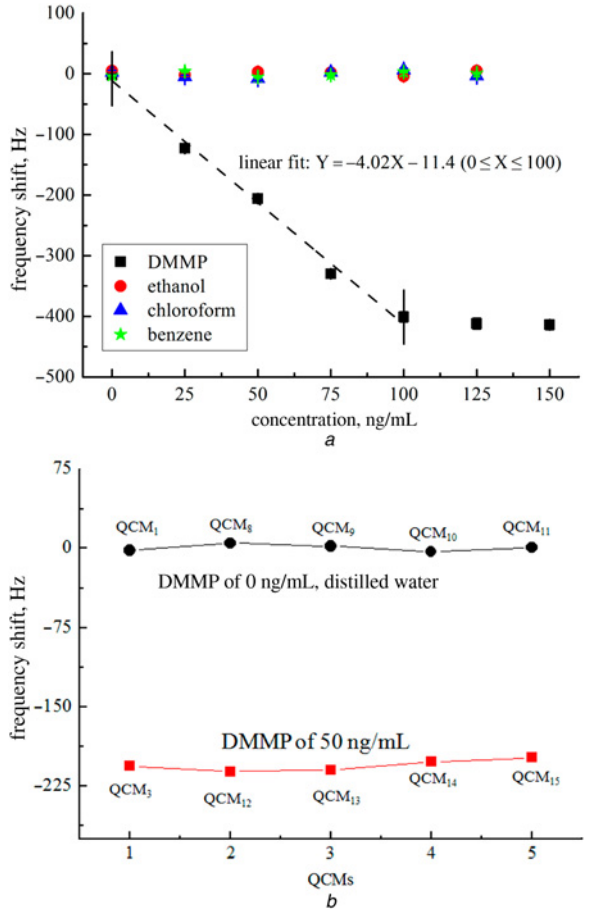

Fig. 4 *Sensing performance of the QCM biosensor*
a Resonant frequency response with different concentrations of DMMP, ethanol, chloroform, and benzene
b Repeatability of the developed biosensor

This adsorption is due to the inhibition reaction between DMMP and AChE. When the inhibition occurred, the main chemical group of DMMP combined with AChE through covalent bond: namely, the phosphorylation of the serine residue in the active site of the enzyme [18]. The detailed chemical reaction equation is shown in Fig. 1. AChE was poisoned, and the mass of the AChE on electrodes increased. Therefore, the resonant frequency of QCM decreased; the higher the DMMP concentration, the greater the decrease of the frequency.

As indicated in Fig. 4*a*, when the concentrations of DMMP exceed 100 ng/ml, the frequency shifts remain almost unchanged. At this concentration, all the immobilised AChE molecules are inhibited, and there are none left to react with DMMP. Moreover, from observation of DMMP test results as shown in Fig. 4*a*, the frequency shift approximately follows a linear relationship with the concentration, and the linear range is from 0 to 100 ng/ml. From the slope of the fitting line, 1 ng/ml changes in concentration of DMMP will give rise to ∼4 Hz variation of frequency on average, thereby indicating a high sensitivity of the developed biosensor. On the basis of the results as shown in Fig. 4*a* and the operational resolution of the frequency counter (1 Hz) applied in the experiment, the LOD for DMMP detection is ∼0.25 ng/ml.

The repeatability and reproductivity of the biosensor were examined by using ten QCMs in Fig. 3. These were all prepared and functionalised with AChE through an identical experimental protocol. Among them, QCM_1 and QCMs_{8-11} were immersed in 0 ng/ml (distilled water) DMMP solution, whereas QCM_3 and QCMs_{12-15} were in 50 ng/ml for 5 min. The results are depicted in Fig. 4*b*. All the frequency changes were around 0 and −200 Hz for detection of distilled water and 50 ng/ml DMMP, respectively, showing a good repeatability of the biosensor. The small variation of frequency changes were due to the different quantities of AChE immobilised on different QCMs. This phenomenon is well consistent with the above-mentioned description of the reaction principle between AChE and DMMP.

Tests of frequency response to ethanol (QCM_8 and QCMs_{16-20}), chloroform (QCM_9 and QCMs_{21-25}), and benzene (QCM_{10} and QCMs_{26-30}) were also carried out to validate the selectivity of the proposed biosensor. No obvious frequency changes except some tiny fluctuations were observed when concentrations increased from 0 to 125 ng/ml for the three contrasting chemicals, as shown in Fig. 4*a*. These tiny fluctuations in frequency can be attributed to uncontrollable factors including accidental fall of dusts, humidity of the surface of QCMs, and manipulation errors.

To better resolve the selective characteristics of the biosensor, we further compared frequency responses to five test samples, as shown in Fig. 5. In this experiment, the concentration of DMMP, ethanol, chloroform, and benzene are all 50 ng/ml. The greatest

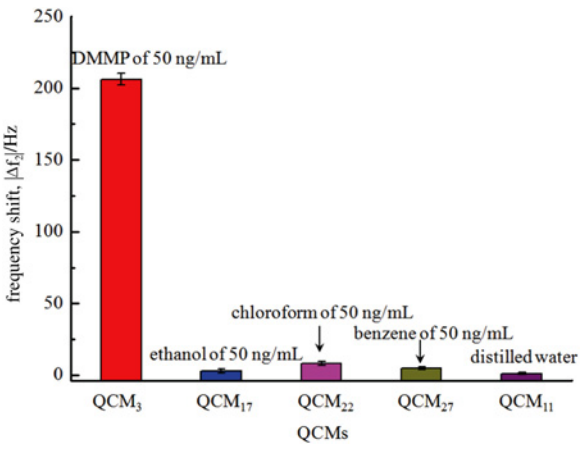

Fig. 5 *Selectivity of the developed biosensor*

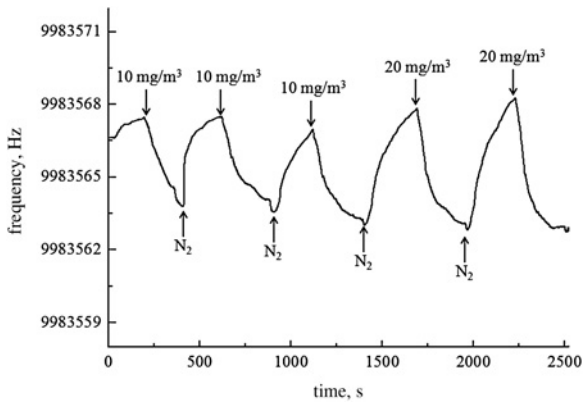

Fig. 6 *Dynamic response for different concentrations of DMMP in gas phase*

response of DMMP further revealed the good selectivity of the developed biosensor. This selectivity in turn indicated the reaction between AChE and DMMP. Due to the fact that there is neither P = O nor P = S functional groups in the other four detected chemicals, frequency responses were almost zero compared with that for DMMP.

In more cases, sarin floats in the air in gas phase during a terrorist attack. Therefore, it is necessary to further validate the feasibility of the biosensor for sensing DMMP in gas phase. The frequency responses were monitored when the modified QCM meets with different concentrations of DMMP gas. Fig. 6 shows the dynamic frequency changes in five test cycles with DMMP concentrations of 10 and 20 mg/m^3 in N_2. As expected, the frequency response for 20 mg/m^3 is greater than that for 10 mg/m^3. Moreover, the response has good recovery. This response recovery shows that the covalent bond between chemical groups of AChE and DMMP will be disconnected under pure N_2. This Letter indicates that the proposed biosensor has great potential in detecting nerve agents in gas phase.

4. Conclusion: In summary, we studied a DMMP biosensor based on QCM transducer and AChE sensitive material. AChE sensitive film was immobilised on the surface of gold electrode of the QCM through cross-linking method. The interaction between AChE and DMMP will change the frequency of QCM, and thus provide the concentration information of DMMP. Liquid DMMP test results prove that the developed biosensor has a high selectivity on DMMP and a good repeatability. In addition, gas DMMP test results indicated a distinct frequency response and a good recovery. Owing to the simple manipulation, low cost, and good frequency response, the developed biosensor has good application potential in public safety and defence construction.

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