Quartz crystal microbalance (QCM) as biosensor for the detecting of *Escherichia coli* O157:H7

Vo Ke Thanh Ngo1,3, Dang Giang Nguyen2, Hoang Phuong Uyen Nguyen2, Van Man Tran1, Thi Khoa My Nguyen4, Trong Phat Huynh2, Quang Vinh Lam3, Thanh Dat Huynh5 and Thi Ngoc Lien Truong6

1 Integrated Circuit Design Research and Education Center (ICDREC), Vietnam National University, in Ho Chi Minh City (VNUHCM), Community 6, Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam
2 Research and Development Center in Saigon Hi-Tech Park, Lot I3, N2 Street, Saigon Hi-Tech Park, District 9, Ho Chi Minh City, Vietnam
3 Faculty of Physics, University of Science, Vietnam National University in Ho Chi Minh City, 227 Nguyen Van Cu Street, District 5, Ho Chi Minh City, Vietnam
4 Faculty of Chemistry, VNUHCM University of Science, Vietnam National University, in Ho Chi Minh City, 227 Nguyen Van Cu Street, District 5, Ho Chi Minh City, Vietnam
5 Vietnamese National University, Ho Chi Minh City, Community 6, Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam
6 School of Engineering Physics, Hanoi University of Science and Technology, 1 Dai Co Viet Road, Hanoi, Vietnam

E-mail: nvkthanh@vnuhm.edu.vn and ngovokethanh@yahoo.com

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Abstract

Although *Escherichia coli* (*E. coli*) is a commensalism organism in the intestine of humans and warm-blooded animals, it can be toxic at higher density and causes diseases, especially the highly toxic *E. coli* O157:H7. In this paper a quartz crystal microbalance (QCM) biosensor was developed for the detection of *E. coli* O157:H7 bacteria. The anti-*E. coli* O157:H7 antibodies were immobilized on a self-assembly monolayer (SAM) modified 5 MHz AT-cut quartz crystal resonator. The SAMs were activated with 16-mercaptopropanoic acid, in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and ester N-hydroxysuccinimide (NHS). The result of changing frequency due to the adsorption of *E. coli* O157:H7 was measured by the QCM biosensor system designed and fabricated by ICDREC-VNUHCM. This system gave good results in the range of 10^2–10^7 CFU mL\(^{-1}\) *E. coli* O157:H7. The time of bacteria *E. coli* O157:H7 detection in the sample was about 50 m. Besides, QCM biosensor from SAM method was comparable to protein A method-based piezoelectric immunosensor in terms of the amount of immobilized antibodies and detection sensitivity.

Keywords: *E. coli* O157:H7, quartz crystal microbalance, self assembled monolayer, piezoelectric sensor, immunosensor

Classification numbers: 2.04, 6.09

1. Introduction

*Escherichia coli* O157:H7 (*E. coli* O157:H7), as one of the most dangerous foodborne pathogens in food industry, is a gram-negative non-spore forming rod and a representative microorganism in the enteric bacteria. This bacterium causes serious illnesses such as bloody diarrhea, bloody feces, anemia and kidney failure [1, 2]. It has been estimated that *E. coli* O157:H7 causes up to eight million deaths worldwide every year from diarrheal diseases [3]. Hence, an establishment of
rapid and sensitive methods for \textit{E. coli} O157:H7 detection is strongly needed to control this pathogenic bacterium in water supplies or food.

Traditional methods for testing of \textit{E. coli} O157:H7 include plating and culturing, enumeration methods and biochemical testing [4]. Although the detection limits for these methods are very low (about a few colony forming units (CFU)/ml), the testing time is time-consuming (from 1 day to 1 week) [5, 6]. Besides, some new techniques for rapid detection of this bacteria have been developed including immunoassays [7], polymerase chain reaction (PCR) [8], DNA microarrays [9], and immunomagnetic separations [10]. It has been shown that sensitivity and selectivity of these methods are good and detection time for these methods is from about 2 h to 24 h [11]. However, these methods have a disadvantage in that they are expensive or complicated due to the use of laboratories equipped with specific instruments and chromospheres. Therefore, they are not suitable for rapid test of \textit{E. coli} O157:H7 bacteria.

In recent years, quartz crystal microbalance (QCM) based biosensor has been a new technology for the rapid detection of pathogens and toxins because of its simplicity in concept, ease of use, low cost, online monitoring, shorter analysis time and suitability for label-free measurement [12]. A QCM biosensor includes an AT-cut quartz crystal wafer sandwiched between two metal electrodes. An applied oscillating electric field induces an acoustic wave. The resonant frequency of QCM is dependent on the mass change at the crystal surface. The relationship between the frequency change and mass loading is shown by the Sauerbrey equation [13]

\[ \Delta f = - \frac{2\Delta m f_0^2}{A\sqrt{\rho_s \mu}}. \]  

where \( \rho_s \) is the quartz density, \( \mu \) is crystal shear module, \( f_0 \) is crystal fundamental frequency of the piezoelectric quartz crystal, \( A \) is crystal piezoelectrically active geometrical area which is defined by the area of the deposited metallic film on the crystal, \( \Delta m \) and \( \Delta f \) correspond to mass and system frequency changes. Based on the combination of QCM devices with highly specific antigen–antibody, enzyme–substrate, and receptors–ligand interaction, QCM biosensor can be used for directly testing the bacteria. Following the Sauerbrey equation, the frequency decrease is proportional to the mass change, which connects to the bacterial concentration [14]. Hence, many researchers applied QCM based biosensor as the transducer to the detection of hygienic important microorganisms as alternatives to the conventional method [15, 16].

Antibody \textit{E. coli} O157:H7 immobilization on the surface of gold electrode is an important work to catch bacteria \textit{E. coli} O157:H7. Until now, many immobilization methods for development of QCM immunosensor in testing \textit{E. coli} O157: H7 are mainly based on polymer membrane [17], Langmuir–Blodgett film [18], protein A [19] and self-assembled monolayer (SAM) [20]. Among these methods, the SAM method presents the simplest way to provide a reproducible, ultrathin and well-ordered functional layer suitable for modification with antibodies, which was responsible for improving detection sensitivity, speed, and reproducibility. Aiming at the gold electrode substrate of QCM (8 MHz AT-cut quartz crystals with diameter about 13.7 mm) for detecting \textit{E. coli} O157:H7, the way to form an SAM with 16-mercaptotrihexadecanoic acid (MHDA), 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC) and ester N-hydroxysuccinimide (NHS) was popularly reported in antibody immobilization, which provides for detecting \textit{E. coli} O157: H7 with a detection limit of \( 10^3 \text{–} 10^6 \text{ CFU ml}^{-1} \) within 30–50 min [14].

In this work we developed a 5 MHz QCM sensor with QCM system designed and fabricated from ICDREC as a biosensor system based on immobilization of the antibodies onto a MHDA-SAM onto gold surface with NHS ester as reactive intermediate for the rapid detection \textit{E. Coli} O157:H7 bacteria as shown by Xiao-Li Su [20]. The immobilization process was optimized to improve the performance of biosensor. The method is simple and fast. Besides, we have a comparison between MHDA-SAM and protein A method for considering the amount of immobilized antibodies and detection sensitivity for testing \textit{E. coli} O157H7.

2. Experimental

2.1. Materials and instrumental

Affinity purified antibodies \textit{E. coli} O157:H7 were purchased from Abcam Company, UK. Protein A-soluble, 16-mercaptotrihexadecanoic acid (MHDA), 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC), ester N-hydroxysuccinimide (NHS) were supplied from Sigma Aldrich (USA). And ethanol, NaOH, aceton, HCl, H2SO4 (98%), H2O2 were purchased were supplied from Sigma Aldrich (USA). And ethanol, NaOH, aceton, HCl, H2SO4 (98%), H2O2 were purchased and were used without treatment from Merck Company (Germany). All the reagents used were AR grade.

We applied a QCM system designed and fabricated by ICDREC-VNUHCM (figure 1) for this study, controlled by a laptop under Windows environment and connected with 5 MHz QCM devices provided by Stanford Research Systems Company. In addition, FE-SEM-MX-51(OLYMPUS Company, Japan) and atomic force microscopy (AFM, Model 5500 AFM system, Agilent Company, USA) were employed to analyze the surface of QCM biosensor.

2.2. Bacteria and culture plating method

\textit{E. coli} O157:H7 as target bacterium was supplied by the Pasteur Institute in Ho Chi Minh City, Vietnam. The bacterial concentration was determined by the conventional surface plating-count method. The culture was then heated in a 100 °C water bath for 15 m to kill all the bacteria, and diluted to the desired concentrations with PBS for further use.
2.3. Method for fabrication of quartz crystal microbalance based immunosensor

2.3.1. Self-assembled monolayer (SAM) method-based immunosensor [14]. The quartz crystal microbalance (QCM) sensors were pretreated with 1 M NaOH for 20 min, 1 M HCl for 5 min in ultrasonic bath and piranha etch solution (H2O2:H2SO4 = 2:3) for 1 min, in sequence, to obtain a clean and highly hydrophobic Au surface. After each pretreatment the QCM sensors were rinsed with ethanol and water successively and dried in a stream of nitrogen.

The pretreated QCM sensors were immersed in an ethanol solution of 200 μl MHDA for 24 h to form a SAM (with one side of the crystal exposed to the solution). After rinsing with ethanol and water, the MHDA-modified crystals were treated with 75 mM EDC and 15 mM NHS for 2 h to convert the terminal carboxylic group to an active NHS ester. After rinsing with water and drying, 100 μl of 0.1 mg ml anti-E. coli O157:H7 antibodies were added onto one side of the QCM sensor and spread over the entire Au electrode for 2 h at 37 °C. The excess antibodies were removed by rinsing with PBS. This crystal was treated continuously with BSA–PBS solution for 1 h to block the untreated and nonspecific sites. After rinsing with PBS and water, the QCM sensors were dried in nitrogen, and finally the sensors were fabricated.

2.3.2. Electrochemical characterization of the gold surface on QCM with SAM. For electrochemical characterization, a conventional three-electrode electrochemical cell was used. A platinum foil of large surface area was used as counter electrode and Ag/AgCl was used as a reference electrode with SAM modified gold QCM electrode as a working electrode. All the experiments were performed at room temperature. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS), which were processed by Biologic MPG2 (Biologic Company, France) were used for the electrochemical characterization of SAMs. CV was performed in a solution of 5 mM potassium ferrocyanide of 50 mV s⁻¹ for 10 scans. The impedance measurements were carried out using an ace signal of 8 mV amplitude at a formal potential of the redox couple using a wide frequency range of 10 Hz to 100 kHz.

2.3.3. Protein a method-based immunosensor. The method for fabricating QCM immunosensor by protein A as described by Babacabm et al [19] is shown as follows: we added about 5 μl of 2.5 mg ml⁻¹ protein A to the pretreated crystals, spread over the entire Au electrodes and stored at 4 °C overnight. The excess protein A was removed by rinsing with PBS. Then, anti-E. coli O157:H7 antibodies with 10 μl of 1 mg ml⁻¹ were added onto gold surface QCM sensor, spread over the entire gold electrodes and kept at 4 °C overnight. The excess antibodies were removed by PBS. Finally, the sample was dried and stored at 4 °C.

2.4. Method for detection of E. coli O157:H7 bacteria by using 5 MHz QCM biosensor

The holder which had the antibodies-treated QCM sensor was a fitted 5 MHz QCM system. Then, the sensor was added with 1 ml PBS solution, while the frequency shift caused by the combination was collected until the curve reached a plateau. During the E. coli O157:H7 detection process, 1 ml of 10⁻¹–10⁷ colony forming units CFU ml⁻¹ bacterial suspension was added into the detection cell for 1 h.

3. Results and discussion

3.1. Characterization of SAM on QCM biosensor

In this work we applied SAM method for the protein linkage interface and used MHDA, a long chain carboxylic acid terminating alkanethiol which was proved to be more stable than other shorter chains [21]. Besides, MHDA in the function of an oriented monolayer on gold surface of QCM sensor was shaped through the strong Au–thiolate bond. In addition, the co-addition of EDC and NHS will improve the stability of the linker compounds by activating the MHDA monolayer and will conjugate E. coli O157:H7 antibody by replacing the active NHS esters through amide bonds [22]. As shown by the Sauerbrey equation, the frequency shift will be deduced from the mass change on the surface. We can calculate the amounts of molecules of each layer on gold surface QCM sensor according to the mass as described by equation [20]

\[ \Delta m = -\frac{A \Delta F}{2.26 \times 10^{-6} \text{F}^{2}}. \]  (2)

Besides, the amounts of molecules \(N\) can be calculated by mass as shown by the following equation

\[ N = \frac{\Delta m}{M_w A}. \]  (3)

with molecular weight \(M_w\) and Avogadro’s constant \(A\) (6.023 × 10²³ mol⁻¹). Substituting expression (2) into...
equation (3) we obtain

\[ N = -2.664 \times 10^{20} \frac{\Delta F}{M_w F^2}. \]  

(4)

The result derived from equation (4) and presented in table 1 showed that frequency shift of QCM crystal changes after each treated step. Based on the observed frequency shift the mass loaded onto the surface crystal was calculated. The frequency shift of MHDA-treated crystals is 322 Hz, equivalent to \(3.853 \times 10^{15}\) MHDA molecules attached onto electrode, density was \(11.983 \times 10^{13}\) MHDA molecules/mm\(^2\), i.e. about 57 ng MHDA mm\(^2\). The mass of MHDA attached onto gold electrode is 3.6 times as many as in the study of Wang’s group [20]. It is explained that the active area of the Au electrode in two studies are different. Our study used a device with the basic frequency of 5 MHz, while Wang and Su [14, 20] used an 8 MHz device. Thus, after immersion in solution of MHDA, density of this molecule is disposed regularly and there were many attached molecules. This means that the immersion time (24 h) was suitable. This result was in agreement with the result recently reported in [14].

Figure 2 indicated that after the MHDA-NHS/EDC treatment of the crystal for 2 h, the difference of its surface in comparison with the gold surface of QCM sensor was almost negligible. But the frequency shift of the MHDA-NHS/EDC treated crystal was very low (97/322 \(\sim 30.12\%)\). It means that NHS/EDC quantity was not more than that of attached MHDA. For example, with the frequency shift of 97 Hz, equivalent to \(2.889 \times 10^{15}\) NHS molecules attached onto electrode, density was \(8.987 \times 10^{13}\) NHS molecules/mm\(^2\), about 17.2 ng NHS mm\(^{-2}\), ratio NHS/MHDA of 75.5% shown that the activating efficiency of NHS is about 75.5%, namely almost 3 MHDA molecules could be activated by 2 NHS molecules (in table 1). Besides, the frequency shift of the immobilized antibodies crystal was about 205 Hz, equivalent to \(4.636 \times 10^{12}\) antibodies anchored. It means that 800 MHDA molecules could be immobilized by 1 antibody. Density of antibody was \(1.442 \times 10^{11}\) antibodies/mm\(^2\), and hence about 36.3 ng mm\(^{-2}\). Antibody attachment is lower than MHDA and NHS. \(N_{\text{Antibodies}}/M_{\text{MHDA}}\) is 0.12%, as good as the ratio in the study of Wang (0.14%). The result of this experiment in QCM system designed and fabricated by ICDREC is as good as the result of Wang [20].

Analyzing the low immobilization efficiency, there are two possible reasons to be considered. First, the volume of anti- \(E.\ coli\) O157:H7 antibody is more gigantic than that of MHDA (about 520 times). Second, many active NHS esters would be hydrolyzed by \(\text{H}^+\) ions in the reagent during the course of immobilization reaction. It is important to adjust the pH value of the antibody solution to the alkalescent level so the OH\(^-\) ions could neutralize the \(\text{H}^+\) ions and inhibit the hydrolyzation of active NHS ester [20].

Figure 3 shows the CV of bare gold QCM electrode and SAMs coated gold QCM electrode in 5 mM potassium ferrocyanide at a potential scan rate of 50 mV s\(^{-1}\). It can be seen from the figure that the bare gold electrode shows a typical CV for the redox couple where the electron transfer reaction is under controlled diffusion. In contrast, the monolayer of the SAM layer modified gold electrode does show a weak peak in the voltammogram since the redox reaction is significantly blocked by the monolayer.

| Layer     | \(M_w\) | \(\Delta F\) (Hz) | \(\Delta m\) (ng) | \(N\)   |
|-----------|---------|------------------|------------------|--------|
| MHDA      | 288.49  | 322              | 1832.5           | 6.4    |
| NHS       | 115.09  | 97               | 553              | 4.8    |
| Antibody  | 150000  | 205              | 1166.6           | 0.0077 |

Table 1. Changes of frequency shift and molecules numbers in each step (QCM system from ICDREC—VNUHCM).
Figure 4. Nyquist plots of the impedance measurement of gold QCM electrode: (●) bare gold QCM, (○) SAM of MHDA, (▲) SAM of EDC/NHS, (✦) SAM of antibody.

Figure 4 shows the impedance plots (Nyquist plots) of the monolayer of SAMs on gold QCM surface in potassium ferrocyanide. The EIS was carried out at a formal potential of $[\text{Fe(CN)}_6]^{3-/4-}$ redox couple. It can be seen from the figure that the bare gold electrode shows a very small semicircle at high frequency region, and a straight line at low frequency region indicates that the electron transfer process of the redox couple is under controlled diffusion. On the other hand, the SAM modified electrode shows the formation of semicircle in the entire range of frequency used for the study, implying a good blocking behavior and complete charge transfer control for the electron transfer process. A very large semicircle obtained in the case of SAM of antibody on gold QCM surface compared to other SAM indicates a high charge transfer resistance and hence an excellent electrochemical blocking ability of the SAM.

Atomic force microscopy (AFM) allows characterizing the organic thin films at a molecular resolution. The surface of the waveguide was then examined by AFM. The topography image on an area of $3 \mu m$, and an example of a roughness profile, are shown in figure 5. A set of several lines drawn in different places of the waveguide and the profile curves show that the surface has an average surface roughness ($R_{\text{rms}}$) of 2.12 nm. The topography image of the sensor surface with a monolayer after binding of MHDA is also presented in figure 5. Examination of the curves of profile at different locations shows that the $R_{\text{rms}}$ is of the same order of magnitude as that of the initial surface, about 5 nm. AFM confirms that the most uniform layer is obtained for a binding time of about 24 h in determined experimental conditions. Thus, for periods of the binding of 24 h, the $R_{\text{rms}}$ is 1.79 nm. This result suggests that the molecules of MHDA bind uniformly on the roughened surface, as well as in the recesses on the bumps, and there was little aggregate.

The results shown by FE-SEM (figure 6), AFM images, electrochemical characterization and frequency shift after each treated step indicated that SAM and anchorage of anti-E. coli O157:H7 antibodies have been successfully performed on a QCM device.

3.2. Influence of PBS solution for testing in 5 MHz QCM system

We measure the frequency shift of QCM immersed in PBS solution for 2 h. At first, there is oscillation because of action on the electrode of PBS. After the establishment of stable surface, the shift barely changes with time. Hence, the baseline in PBS was determined. Results of measurement with PBS show that there was no frequency shift after the establishment of stability (<1 Hz) as antibodies do not capture any antigen. There was no increase of mass on the surface of QCM.

3.3. Investigation of specific antibody E. coli O157:H7 with 5 MHz sensor QCM

Figure 7 shows the result of the test of E. coli O124 suspension in $10^0$–$10^7$ CFU ml$^{-1}$ concentrations, when there was strong frequency change during the first 1000 s. After the time moment when measured system and suspension became stable, the frequency shift reached the baseline (influence of PBS solution), and the frequency did not change. This phenomena should be explained as specific antibodies have not captured any antigen in the suspensions, the mass increase on the SAM layer surface did not take place and there was not any frequency shift. For two remaining control bacteria (Salmonella typhimurium and Bacillus subtilize), results are as good as for E. coli O124. It can be concluded that antibody E. coli O157:H7 is specific.

3.4. Detection of E. coli O157:H7 by SAM method with 5 MHz QCM sensor

Figure 8 shows that in the entire working range of $10^1$–$10^7$ CFU ml$^{-1}$ of E. coli O157:H7, the higher the concentration, the greater the sensor responses. However, the cell concentration was $10^1$ CFU ml$^{-1}$, the temporal response curves could not distinguish from the baseline of negative control (about 4.67 Hz). Because this concentration is very low, bacteria numbers captured by specific antibodies are not enough to make a remarkable frequency change.

Running a sample solution of $10^2$ and $10^3$ CFU ml$^{-1}$ of E. coli O157:H7, frequency shift was about 15 Hz and 34.67 Hz, respectively. This result allows QCM system (figure 1) to be used for qualitative and quantitative analysis of cell concentration in solution. Besides, testing a suspension of $10^3$–$10^7$ CFU ml$^{-1}$ of E. coli O157:H7 for 2 h resulted in frequency decreases which change, respectively, with each other concentration. The higher the cell concentration, the greater the frequency shifts. As more E. coli O157:H7 bacteria have been captured by specific antibodies, the more the mass covered the surface of the Au electrode, and then the more frequency shift of measurement decreased. It takes 50 min to determine frequency shift. This result was as good as the result recently reported [20, 23, 24].

Frequency shift when running a sample solution from $10^2$ to $10^7$ CFU ml$^{-1}$ of E. coli O157:H7 can be distinguished. It means that QCM system, made in ICDREC-VNUHCM, acts well with a detection range of $10^2$–$10^7$ CFU ml$^{-1}$. This also
Figure 5. AFM of the gold surface QCM sensor. (a) Gold surface and (b) MHDA-NHS/EDC binding.

Figure 6. FE-SEM of the gold surface QCM sensor. (a) Gold surface, (b) MHDA-NHS/EDC binding.

Figure 7. Frequency shift of the immunosensor as a function of time in \textit{E. coli} O124 suspension (10^6 CFU ml^{-1}).

Figure 8. Frequency shift of the immunosensor as a function of time in \textit{E. coli} O157:H7 suspension of different concentrations (CFU ml^{-1}) on the QCM system (designed by ICDREC).
proves that sensitivity of the QCM system is similar to few other systems [14, 25].

3.5. Comparison of protein a method-based 5 MHz QCM sensor with SAM method for detection of E. coli O157:H7

A protein A based immunosensor has been developed for detection of E.coli O157:H7 [19]. The SAM-based immunosensor was compared with the protein A in QCM system which was fabricated in ICDREC. As shown in table 2, when testing a sample solution of E.coli O157:H7 in three different concentrations, the frequency shift signals observed by MHDA method are more obvious than that by protein A. It would mean that the peptide binds between specific antibodies and NHS/EDC-MHDA SAM layer are better than those between specific antibodies and protein A.

Figure 9 showed that frequency shift values at any concentration also have remarkable difference. Frequency shift obtained from MHDA-SAM layer method is 2, 1.6 and 2.1 times \((n=3)\), respectively better than that from protein A method at \(10^2\), \(10^4\) and \(10^6\) CFU ml\(^{-1}\). This problem demonstrated that MHDA-SAM layer is more sensitive than protein A method.

Moreover, there is a greater number of antibodies which anchored onto NHS/EDC-MHDA SAM layer than onto protein A. The frequency shift is 322 Hz with MHDA–SAM layer and from 54 to 83 Hz with protein A when the antibodies were immobilized. Therefore, MHDA-SAM method attached antibodies 4.7 times higher than the protein A method.

| Method       | Time | \(10^2\) (CFU·ml\(^{-1}\)) | \(10^4\) (CFU·ml\(^{-1}\)) | \(10^6\) (CFU·ml\(^{-1}\)) |
|--------------|------|--------------------------|--------------------------|--------------------------|
| MHDA         | 1    | 43                       | 67                       |                          |
|              | 2    | 47                       | 70                       |                          |
|              | 3    | 42                       | 72                       |                          |
| Mean         | 14.33| 44                       | 69.67                    |                          |
| Protein A    | 1    | 29                       | 39                       |                          |
|              | 2    | 25                       | 30                       |                          |
|              | 3    | 27                       | 30                       |                          |
| Mean         | 27   | 33                       |                          |                          |

Figure 9. Frequency shift of immunosensor versus time for bacterial concentration by MHDA and protein A methods using QCM system (designed by ICDREC).

system gave good results in the range of \(10^2\)–\(10^7\) CFU ml\(^{-1}\) E. coli O157:H7. Moreover, the results have proved the specificity of the antibodies in the detection of E. coli O157: H7. The time of bacteria E.coli O157:H7 detection in the sample was about 50 m. Besides, SAM method for making a piezoelectric immunosensor is better than protein A method.

4. Conclusions

In this study we succeeded in fabricating an MHDA-SAM immunosensor on gold surface onto QCM sensor with a system made in ICDREC-VNUHCM for rapid and sensitive detection of E. coli O157:H7. The QCM system from ICDREC was successfully applied for the stepwise characterization of the immunosensor and testing E.coli O157:H7. Results showed that NHS ester improved the stability of the linker compounds, and the frequency shifts obtained by 5 MHz QCM sensor were 327, 97, 205 Hz for layers of MHDA, EDC/NHS, and antibody E. coli O157:H7. The

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