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Piezoelectric detection of bilirubin based on bilirubin-imprinted titania film electrode

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ABSTRACT

A novel quartz crystal microbalance (QCM) sensor with a high selectivity and sensitivity has been developed for bilirubin determination, based on the modification of bilirubin-imprinted titania film onto a quartz crystal by molecular imprinting and surface sol-gel techniques. The performance of the developed bilirubin biosensor was evaluated and the results indicated that a sensitive bilirubin biosensor could be fabricated. The obtained bilirubin biosensor presents high-selectivity monitoring of bilirubin, better reproducibility, shorter response time (30 min), wider linear range (0.1–50 μM), and lower detection limit (0.05 μM). The analytical application of the bilirubin biosensor confirms the feasibility of bilirubin determination in serum sample.

Bilirubin, a common metabolite of hemoglobin, is normally conjugated with albumin to form a water-soluble complex and excreted from hepatocytes into bile mainly as bilirubin glucuronides [1–3]. Serum bilirubin can be regarded as an important index for judging liver function and identifying a variety of liver diseases. Extra free bilirubin in body can cause disorders in the metabolism of bilirubin and evoke various diseases [4–6]. Some analytical methods have been developed for bilirubin determination, including enzymatic assays, fluorimetric methods, capillary electrophoresis, high-performance liquid chromatography (HPLC),[1] electrochemical methods, and chemiluminescence [7–12]. These analytic methods have played an important role in bilirubin analysis in the past years.

Molecular imprinting has been recognized as a representative technique for preparing specific binding sites for given molecules in appropriate matrices. In this approach, the shape and functionality of a template can be transcribed onto microporous materials. The configuration of the functional groups in the template may be memorized within the matrix. In the past years, molecularly imprinted polymer has been widely used for chromatographic separation, chemical sensors, enzyme-mimicking catalysts, and biosensors [13–17]. Recently, inorganic matrices have been employed for molecular imprinting. Compared with molecularly imprinted organic polymers, molecularly imprinted inorganic matrices not only possessed a better stability and structural rigidity but also relieved some drawbacks of organic material such as low binding efficiency, slow binding process, and difficult fabrication [18–20]. Silica and titania sol-gel materials have been used to prepare molecularly imprinted thin film, which was used for the determination and separation of organisms [21,22].

The search for highly selective, low-cost, stable, and facile chemical sensors for a biological analyte has attracted a lot of attention. The QCM is a simple, portable, cost effective, high resolution mass sensing instrument, which has been favorably adopted for analytical applications due to its extreme sensitivity to the nanogram level of mass change loaded onto the surface of the QCM resonator. As a mass sensor, QCM has been widely used in biochemistry, environmental, food, life science, and clinical analyses because the instrument provides a label-less method for the direct study of biospecific interaction processes [23–27].

The sensors coated with bilirubin-imprinted polymer film have been fabricated and used for bilirubin recognition [28–31]. However, to the best of our knowledge, it is rarely reported that a sensor modified with bilirubin-imprinted titania film is employed to determine bilirubin. In this study, combining the advantages of high selectivity from piezoelectric microgravimetry using molecular imprinting technique and the high sensitivity from QCM detection, a bilirubin biosensor has been developed for the determination of bilirubin based on the modification of bilirubin-imprinted titania film onto a quartz crystal by surface sol-gel technique.

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[1] *Abbreviations used:* DI, deionized; HPLC, high-performance liquid chromatography; QCM, quartz crystal microbalance; Ti(O–^{n}Bu)$_4$, titanium(IV)-*n*-butoxide.

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Experimental

Materials

Bilirubin, biliverdin, cholesterol, and testosterone were purchased from Sigma Chemical Co., and used without further purification. Titanium(IV)-*n*-butoxide ($Ti(O\text{-}^nBu)_4$ was obtained from Nanjing Youpu Chemical Co., China. All other chemicals were of analytical grade purchased from Shanghai Chemical Co., China. Deionized (DI) water (resistivity of 18 MΩ cm) was obtained from a Milli-Q system (Millipore Inc.), and was used for rinsing and for makeup of all aqueous solutions.

Bilirubin stock solution (1 mM) was prepared daily by dissolving 5.8 mg of bilirubin in 10 ml of 50 mM NaOH solution. Standard solutions of lower concentrations were prepared by further dilution of the bilirubin stock solution. All solutions were stored in amber glass vials wrapped with aluminum foil and placed in the dark to prevent light-initiated bilirubin oxidation. The pH of the solutions was adjusted by adding an adequate amount of dilute HCl and buffered at the desired pH by the phosphate buffer solution. Bilirubin solution (1.0 μM, pH 8.0) was used in our experiments, unless otherwise stated.

Apparatus

QCM measurements were performed using a Q-sense instrument (Gothenburg, Sweden). Mechanically polished AT-cut 9-MHz quartz crystals (diameter of 12 mm, Beijing Chenjing Co., Beijing, China) were vacuum-deposited with gold electrodes (6-mm diameter) on both sides of the surface. To reduce background frequency drift, one electrode was completely insulated from the test liquid. This was accomplished by mounting a glass coverslip over one side of the quartz crystal, which was held in place by two silicon o-rings. The o-rings were so large (10 mm) that they could make contact only with the exposed quartz peripheral to the centrally located electrode and not with the electrode itself. Thus, the covered electrode was in contact only with air. This covering assembly was flexibly fixed and sealed to the flow cell using silicon glue. The electrical contacts were insulated from the test solution using silicon tubing and silicon glue, and only one electrode modified by the bilirubin-imprinted titania film was exposed to the test solution. Water in continuous flow mode was applied to carry the injected sample to the detection position. The working crystal was connected to a transistor-transistor logic integrated circuit (TTL-IC) oscillator (IC 74LS04, Kuan Hsi Co., Taiwan). Both the detector cell and the oscillator were put in a copper faradaic cabin to remove the surrounding electromagnetic noise. The frequency was monitored using a universal frequency counter (Iwatsu SC-7201, Tokyo, Japan) attached to personal computer, and the data were acquired and stored using a computer-based data acquisition system.

IR spectrum was recorded using Fourier transformed infrared spectroscopy (FTIR: Model 883, Perkin-Elmer Optoelectronics Inc., USA).

Preparation of bilirubin-imprinted titania film at the quartz crystal surface

The bilirubin-imprinted titania film was prepared through a surface sol-gel process illustrated in Fig. 1. First, $Ti(O\text{–}^nBu)_4$ (0.8 mmol) and bilirubin (0.5 mmol) were dissolved in 8 mL of toluene–ethanol (v/v, 2:1) mixture, and then vigorously stirred at room temperature until bilirubin was dissolved completely. Subsequently, the solution was diluted by 20 times with water-saturated toluene and continuously stirred for 5 h to get a sol-gel solution which was used as a dipping solution. The quartz crystal was immersed into the dipping solution for 5 min at room temperature, rinsed with toluene, hydrolyzed in water, and dried in N_2 gas. These steps, which constituted one cycle of chemisorption and activation, resulted in the incorporation of bilirubin into titania thin film. The thickness of imprinted film can be controlled by the number of "dipping-rinse-hydrolyzation-drying" cycles, and estimated according to the amount of titania at the surface of the quartz crystal. The film with around 0.8 μm thickness was used in our experiments, unless otherwise stated. In order to form imprinted sites for template molecules, the bilirubin-incorporated film was treated with ammonia aqueous solution (1 wt%) and rinsed with DI water. The procedures were repeated until a constant frequency was obtained. As a result, the template molecules were eliminated, and a titania thin film with cavities imprinted with bilirubin molecules was formed.

The nonimprinted titania film was prepared using the same procedure except for no addition of bilirubin template.

QCM measurements

To monitor the *in situ* response of the bilirubin biosensor to the bilirubin molecule, the following procedure was adopted. After mounting the crystal in the cell, a small amount of aqueous solution was introduced into the cell by a peristaltic pump Ismatec (IPC-8 model) with a constant flow rate. When the frequency became stable, the solution containing bilirubin was injected into the cell within 5 s. Time-dependent change in the frequency was recorded continuously by a frequency counter and stored in a microcomputer during the reaction process of bilirubin.

Results and discussion

FTIR characterization

The molecularly imprinted titania film at the surface of the quartz crystal was prepared by the molecular imprinting technique and surface sol-gel process. The direct evidence for the incorporation and removal of bilirubin in the titania matrix was obtained by FTIR spectroscopy. Fig. 2 shows the FTIR spectra of pure titania, pure bilirubin, and bilirubin-imprinted titania. Compared with the pure titania, new adsorption bands appeared in the infrared adsorption spectrum of bilirubin-imprinted titania (see curve 'c' in Fig. 2). The characteristics of the new adsorption bands were quite different from those of pure titania, but similar to those of pure bilirubin. These new bands at 3400, 3261, 2913, 1696, 1647, 1612, 1249, 989, and 945 cm^{-1} were assigned to the NH stretching mode, asymmetric OH stretching, asymmetric CH stretching in the CH_2 group, the carboxylic C=O stretching mode, C=O stretching mode, C=C stretching vibration, C—C and C—N bonds, the methyl groups, C—H bending mode, respectively. The results are in good agreement with the characteristic absorption bands of bilirubin; the only difference is that the absorption band of bilirubin at 3261 cm^{-1} (asymmetric OH stretching) disappears in the absorption spectrum of the bilirubin-imprinted titania, which can be attributed to the hydrogen interaction between bilirubin molecules and titania. This is direct experimental evidence indicating that bilirubin reacted with titania to form a complex and was incorporated into the titania film in the process of the molecular imprinting. These spectral features due to bilirubin incorporation completely disappeared after the bilirubin-imprinted titania film was rinsed with ammonia solution (see curve 'd' in Fig. 2). However, it was observed that the characteristic bands of bilirubin were again evident when the bilirubin-imprinted titania film was again immersed in bilirubin solution and washed with DI water (see curve 'e' in Fig. 2). Therefore, it was suggested that the treatment

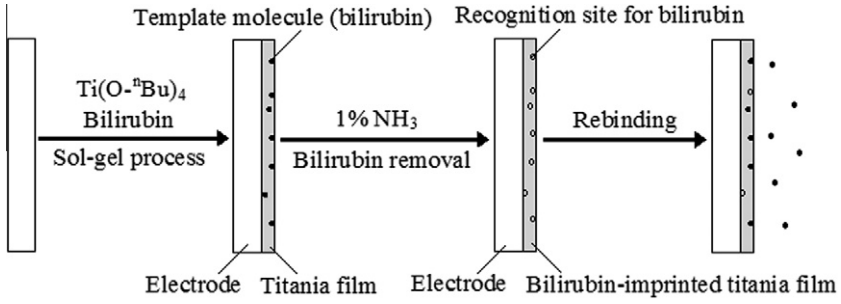

Fig.1. Schematic illustration of the fabrication of the bilirubin-imprinted titania film at the surface of a quartz crystal.

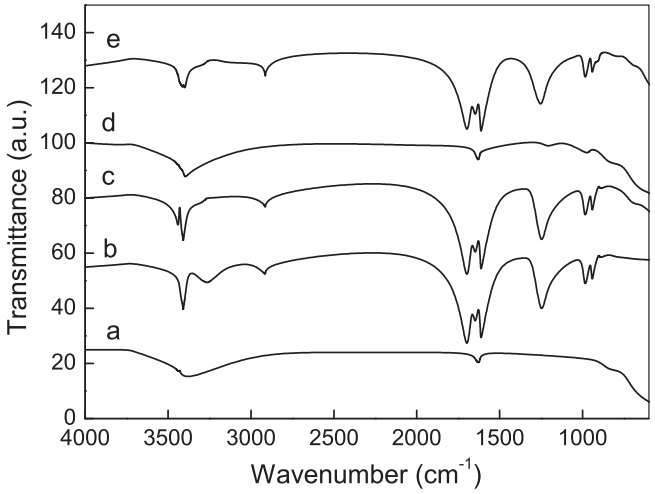

Fig.2. FTIR spectra of (a) pure titania, (b) pure bilirubin, (c) bilirubin-imprinted titania before washing with ammonia solution, (d) bilirubin-imprinted titania after washing with ammonia solution, and (e) titania imprinted after bilirubin rebinding.

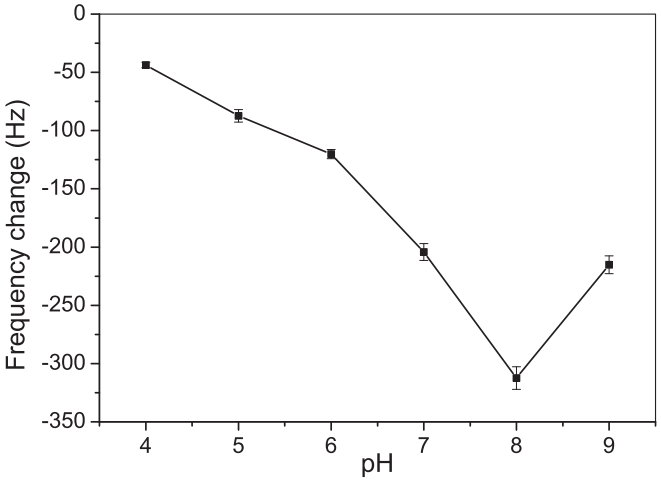

Fig.3. Effect of pH on frequency response of the bilirubin biosensor. 1.0 μM bilirubin, 25 °C, average of three experiments.

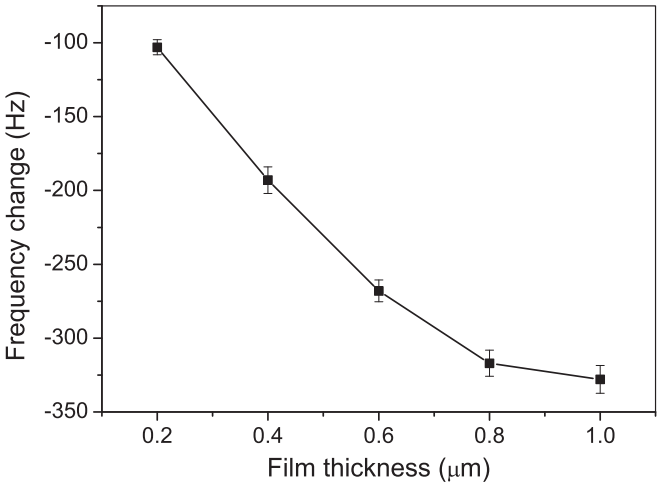

Fig.4. Effect of imprinted film thickness on frequency response of the bilirubin biosensor. 1.0 μM bilirubin, pH 8.0, average of three experiments.

of the electrode with ammonia solution would destroy bilirubin incorporated in titania matrix and resulted in the formation of recognition sites for bilirubin molecule.

Response characteristics of the bilirubin biosensor

Effect of pH and imprinted film thickness on response of the bilirubin biosensor

The frequency response of the bilirubin biosensor depends on the pH of bilirubin solution. As seen in Fig. 3, the value in frequency change increased sharply when pH was varied from 4 to 8, while decreased at pH above 8. Such phenomena can be explained as follows: in the pH range of 4–8, the predominant titania surface group is >Ti—OH and the main functional group of bilirubin is BH^-; the interaction between titania and bilirubin mainly depends on the hydrogen bonding; the amount of the main groups in titania and bilirubin will increase with the increasing pH; thus, the bonding force will be enhanced and an increasing adsorption of bilirubin will be observed. At pH above 8, the predominant surface group of titania is >Ti—O^- and the main form of bilirubin is B^{2-}; therefore, the interaction between titania and bilirubin is weakened due to the same negative change, causing a decreased adsorption of bilirubin [32]. According to the above analysis, the optimum pH for the sensor response was found to be 8.0, and all further experiments were carried out at pH 8.

The imprinted film thickness has a significant effect on response of the bilirubin biosensor. As seen in Fig. 4, the value in frequency change increased with the increasing thickness of imprinted films, indicating that bilirubin molecules can enter into the inner layer of bilirubin-imprinted titania film due to the high porosity of the imprinted titania film with channels and holes. However, when the imprinted film thickness was above 0.8 μm, a further increase in the imprinted film thickness caused a slight enhancement of frequency response. Such result may be attributed to the hindrance of titania matrix in imprinted film, making it difficult for bilirubin to enter into the inner layer of thicker titania film. Therefore, the

bilirubin-imprinted titania film with 0.8 μm thickness was used in our experiments.

Response time, linear range, and detection limit

Fig. 5 shows the typical frequency response versus time profile of the bilirubin biosensor modified with bilirubin-imprinted titania film in 1.0 μM bilirubin solution (pH 8.0) at 25 °C. The steady-state response was reached in about 30 min after injection of the bilirubin solution. It is believed that mass transfer resistance by the binding cavity, the titania matrix, and strength of the interaction could be major factors affecting the response tine of the bilirubin biosensor.

The dynamic range of the bilirubin biosensor was studied by monitoring the bilirubin solution in the concentration range from 0.05 to 80 μM. Fig. 6A presents the plot of frequency response versus bilirubin concentration. Over the low concentration range (0.1–50 μM), good linearity was obtained (see Fig. 6B). The linear regression equation was y (Hz) = −157.4–91.8 [bilirubin] (μM), $R = 0.9988$, $n = 8$. When the bilirubin concentration was higher than 50 μM, the frequency response started to level off with the increase of bilirubin concentration. The sluggish frequency change for the higher bilirubin concentration can be attributed to the kinetic restriction of the bilirubin reaction involved. When the concentration of substrate was overloaded, the original first-order reaction would be changed to a zeroth-order reaction on which the reaction rate became independent of the substrate concentration. The detection limit of the bilirubin biosensor was found to be about 0.05 μM (S/N = 3), which was lower than that reported by other researchers [28,29]. Such phenomena can be attributed to the strong adsorption of bilirubin-imprinted titania film and extreme sensitivity of QCM.

Selectivity

The selectivity is an important parameter in evaluating sensors. In order to better study the selectivity of the bilirubin biosensor modified with bilirubin-imprinted titania film, biliverdin, cholesterol, and testosterone were chosen as competitive adsorption molecules for comparison. Fig. 7 shows the chemical structures of bilirubin, biliverdin, cholesterol, and testosterone. Biliverdin has almost the same chemical structure and molecular weight as bilirubin. Cholesterol and testosterone were chosen for comparison because they, together with bilirubin, are always found in the serum. Therefore, to know how they interfere with the binding of bilirubin by imprinted titania film is essential for the in vitro measurement of serum samples [33]. Fig. 8 shows the frequency change caused by adsorption of bilirubin, biliverdin, cholesterol, and testosterone on the imprinted and nonimprinted titania film. Compared with the nonimprinted titania film, the bilirubin-imprinted titania film exhibited a higher response to template molecules under the same experimental conditions. The values of frequency change caused by bilirubin, biliverdin, cholesterol, and testosterone on various films were recorded in detail and the obtained results are tabulated in Table 1. According to the frequency change, the relative selectivity coefficients of bilirubin-imprinted titania film for bilirubin/biliverdin, bilirubin/cholesterol, and bilirubin/testosterone pairs were 2.8, 3.1, and 1.2 times greater than that for non-imprinted titania film, respectively, indicating that the bilirubin-imprinted titania film had a higher adsorption and selectivity to bilirubin molecules. These results confirm strongly that a sensor with high selectivity for bilirubin determination is obtained in our experiments. This means that bilirubin can be determined by the sensor modified with bilirubin-imprinted titania film even in the presence of other interferents.

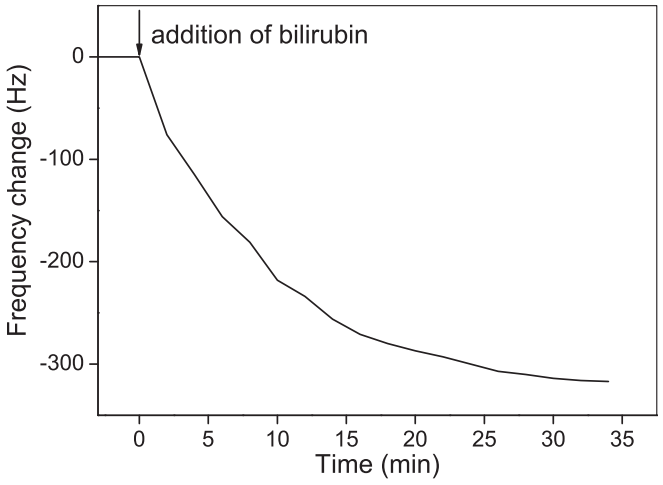

Fig.5. Response of the bilirubin biosensor to 1.0 μM bilirubin solution (pH 8.0, 25 °C).

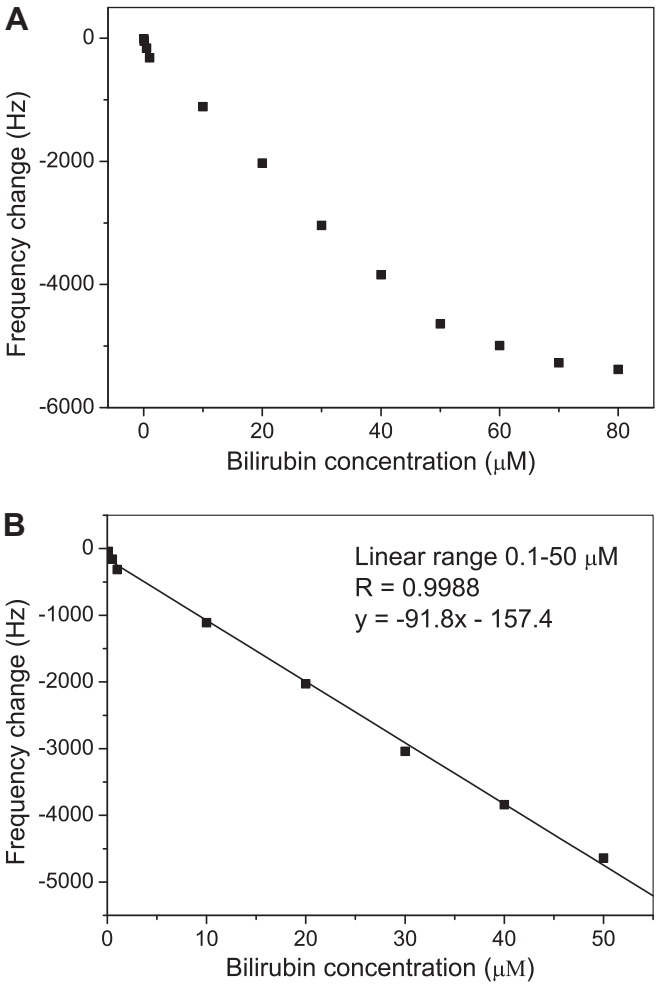

Fig.6. (A) Calibration graph obtained for the bilirubin biosensor in a bilirubin concentration range from 0.05 to 80 μM. (B) The linear part of the calibration graph. Experimental conditions: pH 8.0 and 25 °C.

Reusability and analytical application

The regeneration of the bilirubin-imprinted titania film is of critical importance for application as a sensor. Reproducibility of

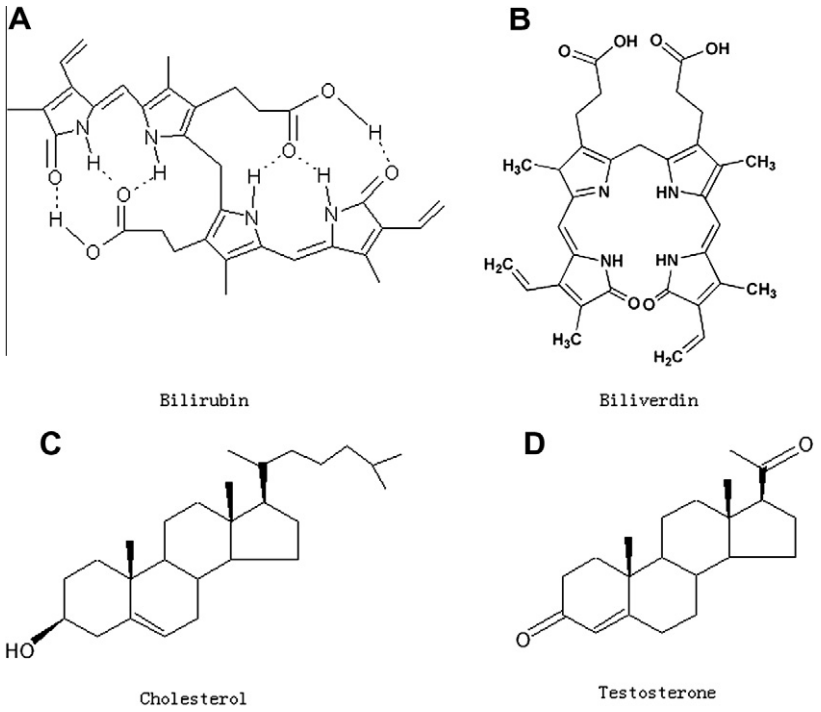

Fig.7. Chemical structures of (A) bilirubin, (B) biliverdin, (C) cholesterol, and (D) testosterone.

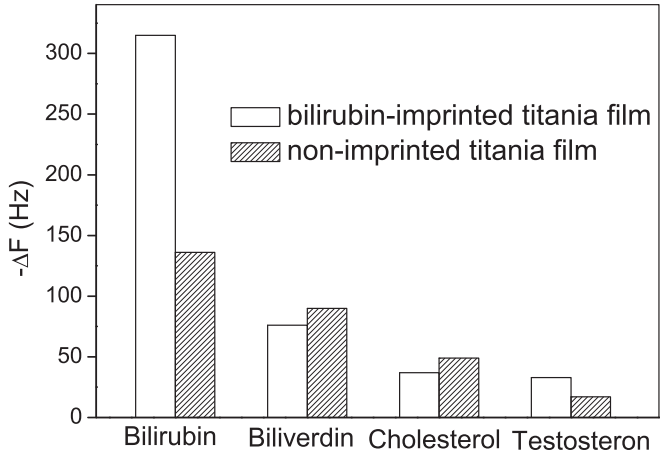

Fig.8. Comparison of adsorption selectivity of the QCM sensors. Concentration 1.0 μM, pH 8.0, and temperature 25 °C.

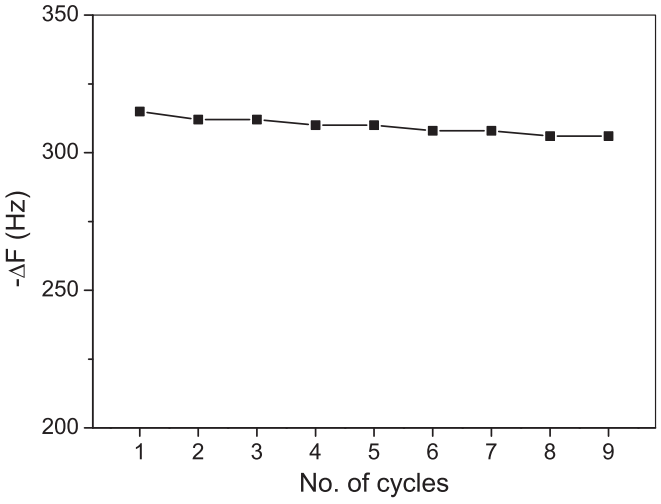

Fig.9. Reproducibility of frequency response from the same bilirubin biosensor modified with bilirubin-imprinted titania film in 1.0 μM bilirubin solution (pH 8.0) at 25 °C.

Table 1
The frequency change caused by bilirubin, biliverdin, cholesterol, and testosterone on imprinted and nonimprinted titania films.

Films	Frequency change (Hz)				Relative coefficient		
	BR	BV	CH	TS	BR/BV	BR/CH	BR/TS
Imprinted titania film	−315	−76	−37	−33	4.14	8.51	9.55
Nonimprinted titania film	−136	−90	−49	−17	1.51	2.78	8.00

BR, bilirubin; BV, biliverdin; CH, cholesterol; TS, testosterone.

the same bilirubin biosensor was evaluated by measuring the response signal nine times in a continuous manner and the obtained results are presented in Fig. 9. After each injection of bilirubin solution and the attainment of equilibrium, the bilirubin biosensor was recovered by sequential washes with ammonia aqueous solution (1%) and DI water until the frequency of QCM reached a steady value. The sensor can be stored in DI water at room temperature when not in use. It was observed from Fig. 9 that the bilirubin biosensor exhibited better reproducibility, and the response signal of the sensor was about 2.9% loss at the 9th cycle. In addition, our experiments indicated that the bilirubin biosensor was quite stable not only when operated for a long period under a continuous flow detection system but also when reused from time to time. The sensor has proved its stability for more than 3 months with a slight loss of sensitivity. Hence, it can be concluded that the bilirubin biosensor can be used many times without a significant decrease of response signal.

To demonstrate the feasibility of the bilirubin biosensor for analytical application, the recovery test of the bilirubin biosensor was

Table 2
Recovery test of the bilirubin biosensor.

Bilirubin concentration (μM)		Recovery (%)
Added	Found[a]	
0.50	0.48 ± 0.01	96.00
5.00	4.95 ± 0.22	99.00
15.00	15.06 ± 0.35	100.40
30.00	29.38 ± 1.00	97.93
40.00	40.23 ± 1.23	100.58

[a] Average of three measurements ± SD.

performed by the standard addition method. As seen in Table 2, with five different additions of bilirubin to the serum sample, the obtained recoveries ranged from 96.00 to 100.58%. The high recovery indicates that the bilirubin biosensor is feasible for practical analysis. It is believed that a reliable sensor has been obtained for bilirubin determination in serum sample in our study.

Conclusions

The bilirubin-imprinted titania film at the surface of quartz crystal was prepared by molecular imprinting and surface sol-gel techniques. By using the bilirubin-imprinted titania film as the recognition material, a novel QCM sensor with good reproducibility, short response time, wide linear range, low detection limit, and high selectivity has been developed for the determination of bilirubin. The detection of bilirubin by the bilirubin biosensor is rapid and sensitive in the detection system. The analytical application of the bilirubin biosensor demonstrates the feasibility of bilirubin determination in serum sample.

Acknowledgments

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