

Synthesis of bilirubin imprinted polymer thin film for the continuous detection of bilirubin in an MIP/QCM/FIA system

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Bilirubin, a lipophilic and cytotoxic yellow-orange pigment, is produced from hemoglobin metabolism of aged red blood cells. It is transported to the liver as a complex with albumin and excreted into the bile as bilirubin glucuronides. Bilirubin can be regarded as an important index for judging liver functions and is used to identify a variety of liver diseases. Disorders in the metabolism of bilirubin may cause a yellow discoloration of the skin and other tissues, called hyperbilirubinemia. High bilirubin concentration may even cause hepatic or biliary duct dysfunction, and also permanent brain damage or death in the most severe cases. In this work, a bilirubin imprinted polymer (BIP) film with a thickness of approximate 150 nm was coated on a thiol pretreated Au electrode of a quartz crystal microbalance (QCM) chip. The BIP thin film was synthesized using 4-vinylpyridine (4-Vpy) as the monomer, divinylbenzene (DVB) as the cross-linker, and benzophenone as the initiator. By using a photo-graft surface polymerization technique with irradiation by ultra-violet light, a thin BIP film was prepared, from which a biomimetic sensor for the detection of bilirubin was developed. The sensor was able to discriminate bilirubin in solution owing to the specific binding of the imprinted sites. The BIP/QCM chip has been repeatedly used for more than 7 months in many continuous experiments. The detection signal of bilirubin from the BIP thin film/QCM was compared with the non-BIP thin film/QCM. A binding capacity of 62.0 mg/g MIP could be achieved. The imprinted ratio was 10.46. Biliverdin, an analogue of bilirubin, was used for comparison. The analogue comparison confirmed the binding specificity of the BIP film toward bilirubin. The selectivity achieved can be as high as 31.2. The pH of the analyte solution affected the detection frequency as well as response time. With proper solvent for elution and recovery, flow injection analysis (FIA) could be applied. The combination of molecular imprinting and a piezoelectric quartz crystal microbalance was shown to function as a simple, specific, and reusable bio-sensing system. The chip had an excellent storage stability and the sensing unit could be regenerated for reuse for a long period of time. The performance of the BIP/QCM chip was evaluated of reproducibility from single chip and different chips and considerable success was achieved. A linear calibration of the bilirubin concentration with respect to the frequency shift was successfully obtained from the BIP/QCM/FIA system. The reproducibility of measurements from the same BIP/QCM chip was confirmed. In addition, repeated detection was also confirmed from different BIP/QCM chips. In conclusion, a combined BIP thin film/QCM/FIA method was successfully established for the detection of bilirubin concentration using a molecularly imprinted film.



The functional monomer was 4-vinylpyridine (4-Vpy). The initiator for the polymerization was

benzophenone. The cross-linking agent was divinylbenzene (DVB). α -Bilirubin was the target compound in this study. The thiol compound, allyl mercaptan, was used for surface modification of gold electrode. A gold electrode surface was sputtered on both faces of a QCM chip. Prior to the detection, the QCM chip had to be washed. The surface of the cleaned gold electrode of the QCM chip was then modified with a layer of self-assembled allyl mercaptan. Benzophenone solution, as the initiator, was dropped on the allyl mercaptan modified gold electrode surface. Thus, a thin layer of initiator as graft was fabricated on the surface. 4-Vpy and DVB were added into the bilirubin solution. The above solution was then spread on the modified surface of the electrode. The electrode chip was placed in a glass chamber with N₂ flow. Polymerization was then initiated by UV irradiation. After the photo-graft surface polymerization was completed. The electrode coated with the bilirubin imprinted poly(4-Vpy-co-DVB) film was repeatedly washed to remove the template as well as possible residual chemicals. Non-imprinted poly(4-Vpy-co-DVB) film was prepared in the same way except that there were no bilirubin template molecules added. Chemical structures of 4-Vpy monomer, DVB crosslinker, benzophenone initiator, and allyl mercaptan are plotted in Fig. 1. The schematic representation for the synthesis of MIP film on the Au surface of a QCM chip is illustrated in Fig. 2.

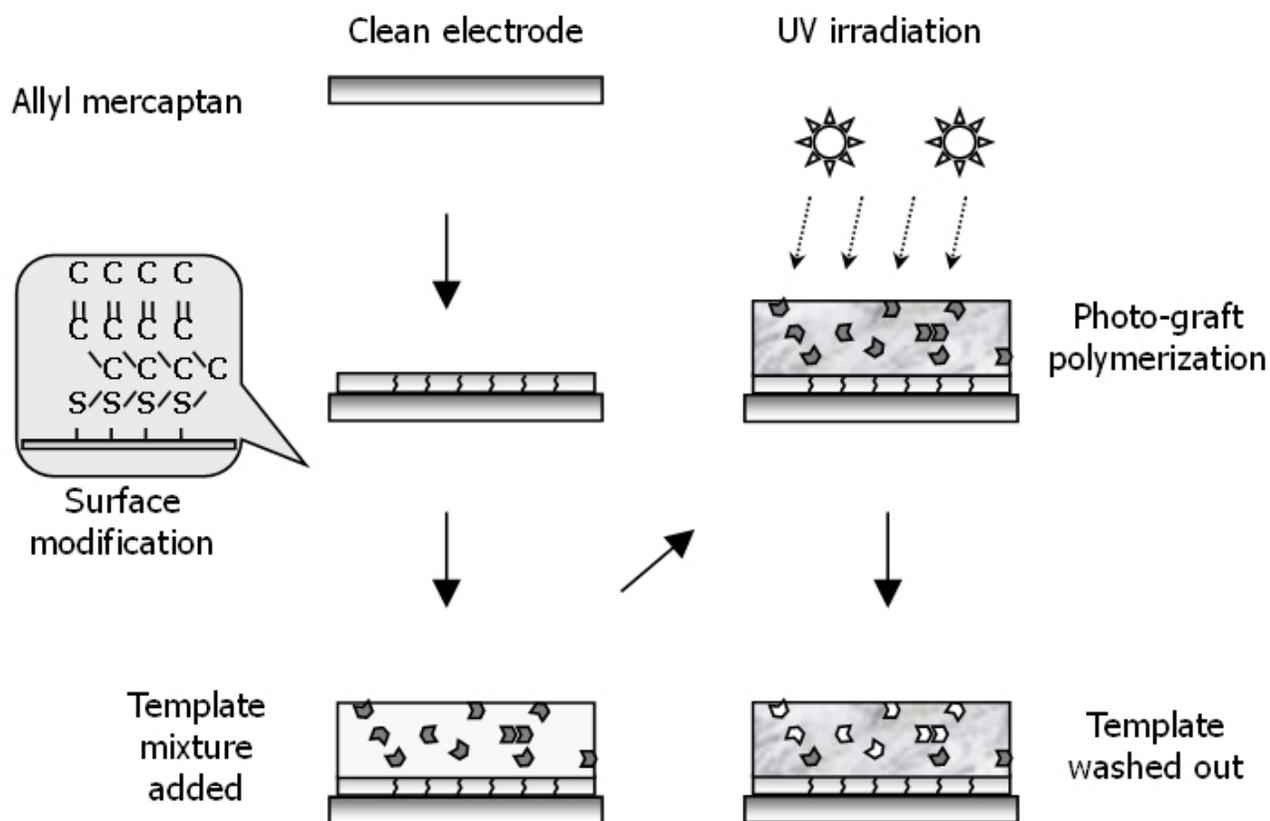
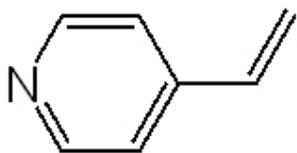


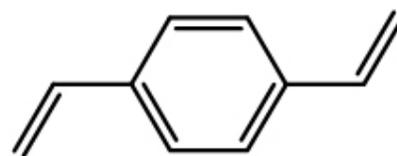
Figure 1 Schematic representation for the preparation of molecularly imprinted polymer film onto the Au surface of a QCM chip.

(a)



Functional monomer
4-vinylpyridine

(b)



Cross-linker
Divinylbenzene

(c)

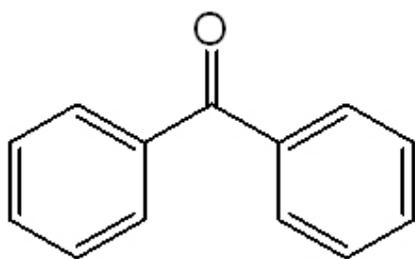


Photo-initiator
Benzophenone

(d)



Allyl mercaptan

Figure 2 Chemical structures of: (a) 4-Vpy monomer; (b) DVB crosslinker; (c) benzophenone initiator; (d) allyl mercaptan.

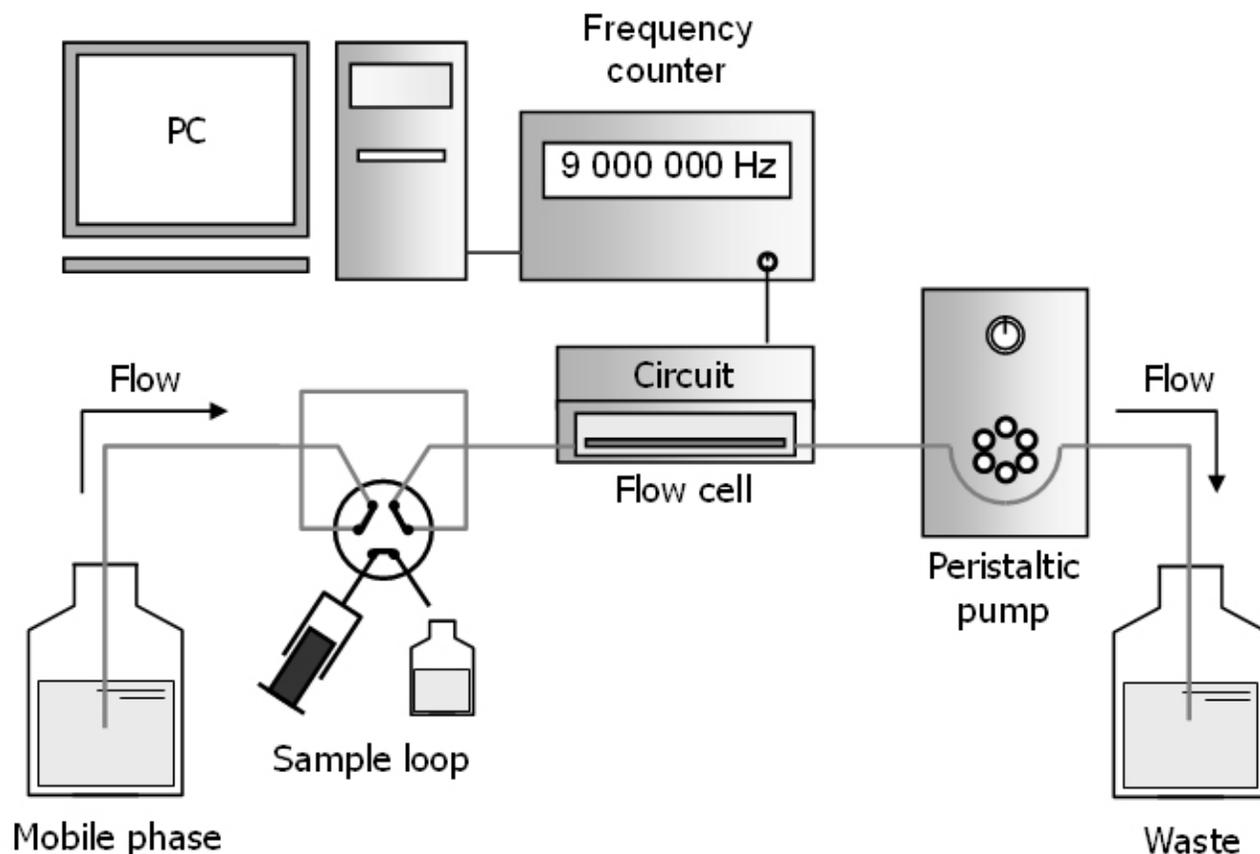


Figure 3 Setup of the MIP/QCM/FIA system.

The detection of bilirubin concentration from the oscillation frequency change of the QCM chip coated with MIP thin film was carried out by a QCM instrument. Sample was injected to the system. Water in continuous flow mode was applied to carry the injected sample to the detection position, which is called flow injection analysis (FIA). The setup of the MIP/QCM/FIA system is illustrated in Fig. 3. The quartz crystal chip coated with a BIP thin film was placed in the holder of the QCM instrument. The FIA system was turned on and used with a continuous water flow rate. The flow was continuously delivered into the flow channel until a stable response of frequency was reached. Then, analyte solution was injected into the flow system. The frequency change was recorded until another stable frequency was reached. After each detection, a continuous flow of water was switched to carry washing solvent through the flow channel. Thus, the QCM chip was in situ regenerated.

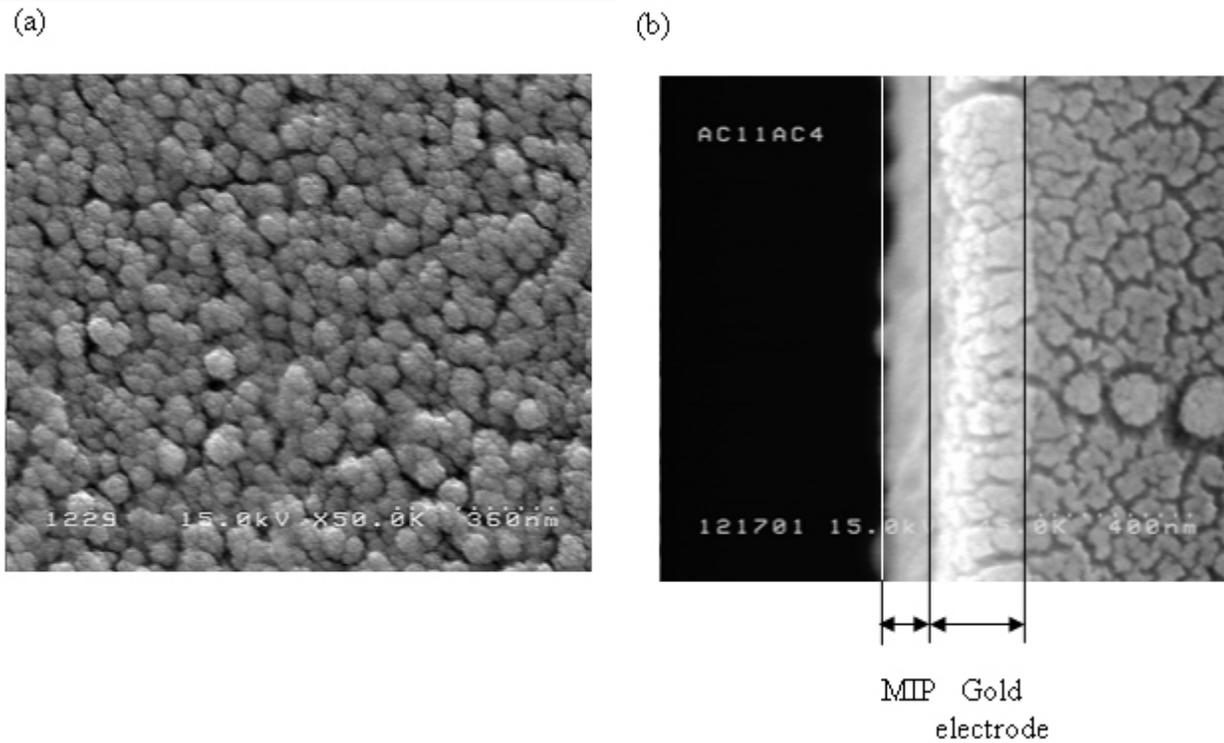


Figure 4 SEM photos of the surface of the synthesized BIP film. (a) top view; (b) side view.

The Au electrode surface of the QCM chip was modified by allyl mercaptan to improve the adhesion between the BIP film and the gold electrode surface. Figures 4(a), 4(b) are the SEM photos for the top view and side view of the BIP film coated on the Au electrode. A homogeneous surface of the BIP film is shown in Fig. 4(a). In Fig. 4(b), the thickness of the BIP film was estimated to be approximate 150 nm from the scale bar index of the photo. A relative smooth film surface can also be observed in the photo.

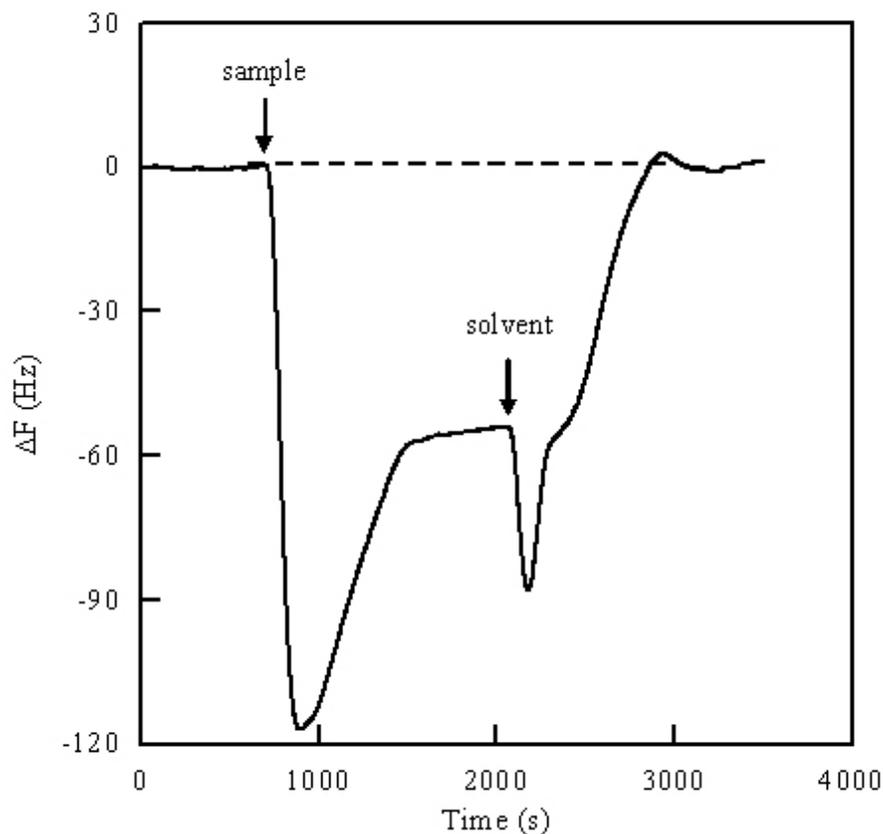


Figure 5 A representative response curve of frequency change with respect to sample and solvent injections.

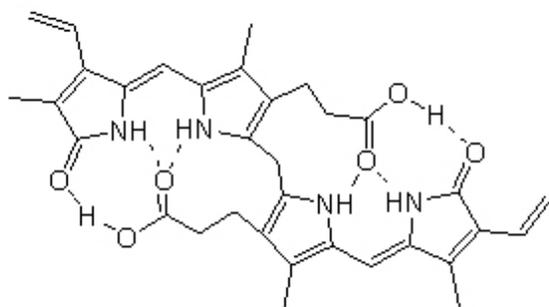
After the BIP film was prepared, the QCM system was used to monitor the washing condition of the film. The detection of bilirubin concentration in solution was performed by a BIP/QCM system with a flow injection loop. Water as the mobile phase was continuously delivered through the channel of the system. Samples were injected into the system from the sample loop. Fig. 5 was a typical signal of QCM detection. Sample injection caused a frequency shift, which was a result from the binding of the analyte solution to the BIP film. After the frequency reached a stable level, the eluent was injected into the flow system to wash off the adsorbed analyte. It was observed from the figure that the decreased frequency after sample injection could recover to the original level after the eluent was applied.

Table 1 Response time of the QCM/FIA system versus pH.

pH of bilirubin solution	QCM response time (min)	Relative square deviation (RSD)
5.65 ± 0.25	11.56 ± 0.24	3.0%
7.20 ± 0.50	19.00 ± 1.20	8.9%
8.30	24.10	
8.90 ± 0.20	42.05 ± 1.25	4.2%
10.70	47.70	
11.45 ± 0.05	53.25 ± 0.25	0.7%

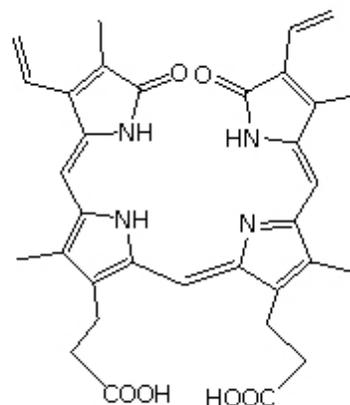
The effect of pH on QCM detection of bilirubin solution was examined in the range from of 5.4 to 11.5 and the response time corresponding to pH is listed in Table 1. When bilirubin solution of higher pH was applied, the response time was longer. The response time may correspond to the interaction between the analyte and the BIP film. At higher pH values bilirubin had stronger binding strength to the MIP film, therefore, a longer time was required to reach equilibrium. The chip was further examined for its calibration of bilirubin concentration. A linear correlation of bilirubin concentration versus frequency shift with a slope of 23.45 (Hz/mg/dl) was obtained. The calibration range was between 0.45 to 11.0 mg/dl. The detection of bilirubin concentration could then be carried out with the linearly calibrated correlation of bilirubin concentration from the QCM. A maximum binding capacity of approximate 62.0 mg/g MIP was estimated.

(a)



Bilirubin
MW 584.7

(b)

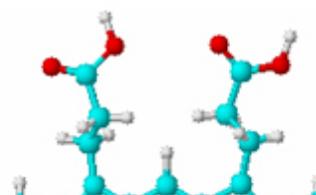


Biliverdin
MW 582.7

(c)



(d)



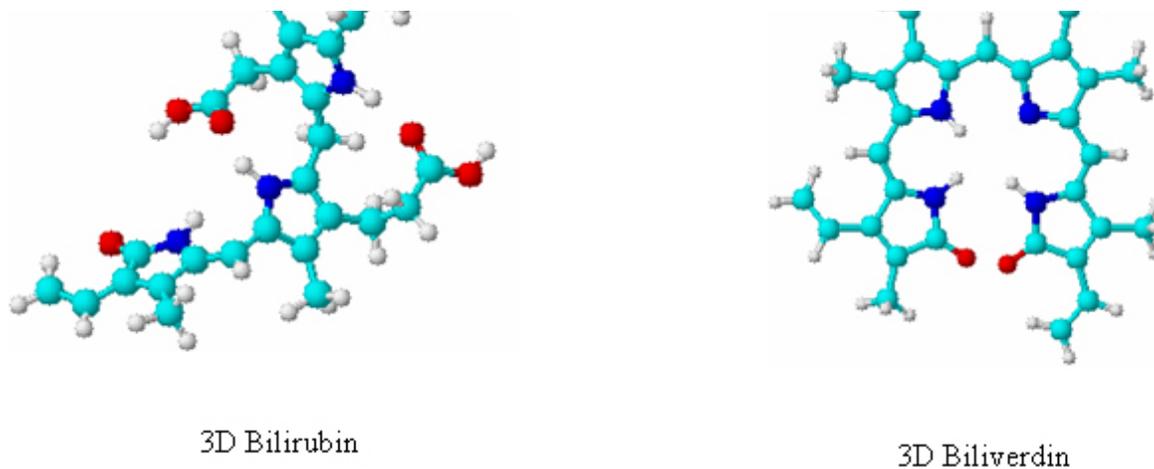


Figure 6 Chemical structures of: (a) bilirubin; (b) biliverdin and 3D-conformation of (c) bilirubin; (d) biliverdin.

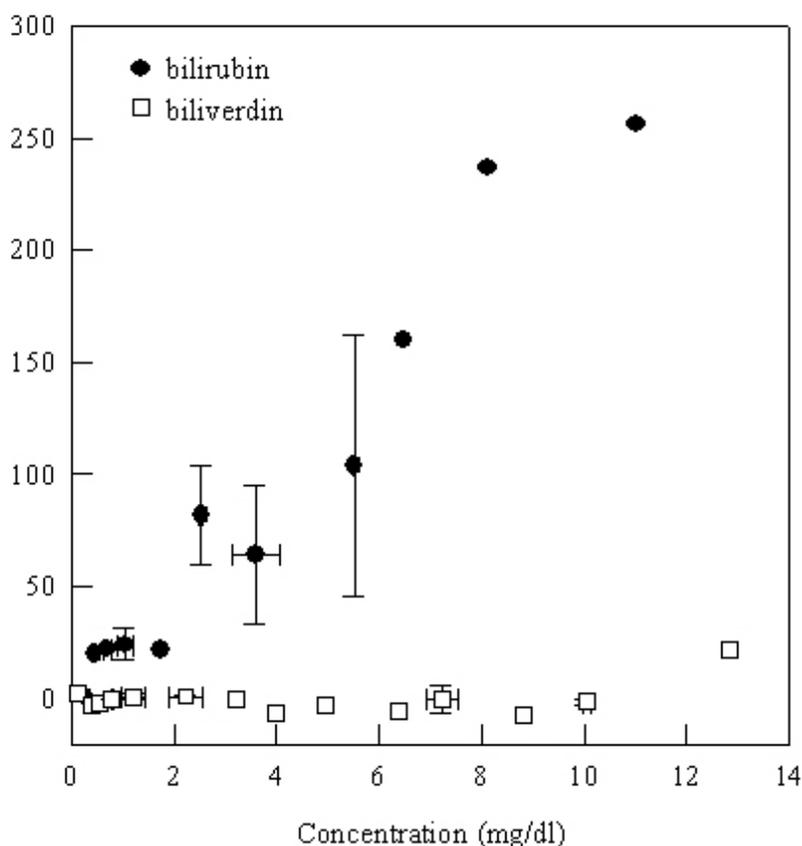


Figure 7 Comparison of the response frequency profiles for the detection of bilirubin and biliverdin from the BIP/QCM chip. The average RSD of bilirubin is 32.46%.

The selectivity of the BIP/QCM chip was also tested. Biliverdin, an analogue to bilirubin, was chosen for comparison. The chemical structures and molecular weights of bilirubin and biliverdin are almost the same as shown in Fig. 6. The major difference between these two molecules occurs at position C10, with a single bond in bilirubin and a double bond in biliverdin. Hence, bilirubin forms intramolecular hydrogen bonds while biliverdin does not. Fig. 7 is the detection result compared to the BIP/QCM chip. The average relative square deviation (RSD) of bilirubin was 32.46%. For biliverdin, its mean value was extremely small and thus the value of the corresponding RSD became large and no longer made sense.

The detection signal of biliverdin showed that the BIP film favored bilirubin much more than biliverdin and therefore the binding amount of bilirubin by the BIP film was much higher than that of biliverdin. Obviously, the plot revealed that BIP was selective and showed binding specificity toward bilirubin. The maximum selectivity of the BIP film prepared on the QCM chip, defined as the binding amount of bilirubin to that of biliverdin, was 31.20. A BIP film and non-imprinted polymer (non-MIP) film were synthesized on QCM chips, respectively. Fig. 8 is the detection results of bilirubin concentrations from BIP/QCM and non-MIP/QCM chips. The imprinted polymer film showed a significant binding effect to bilirubin whereas the non-imprinted polymer film showed less interaction. The maximum imprinted ratio, defined as the binding capacity of bilirubin to that of biliverdin, was approximate 10.46. This result confirmed the molecular imprinting effect in the BIP film.

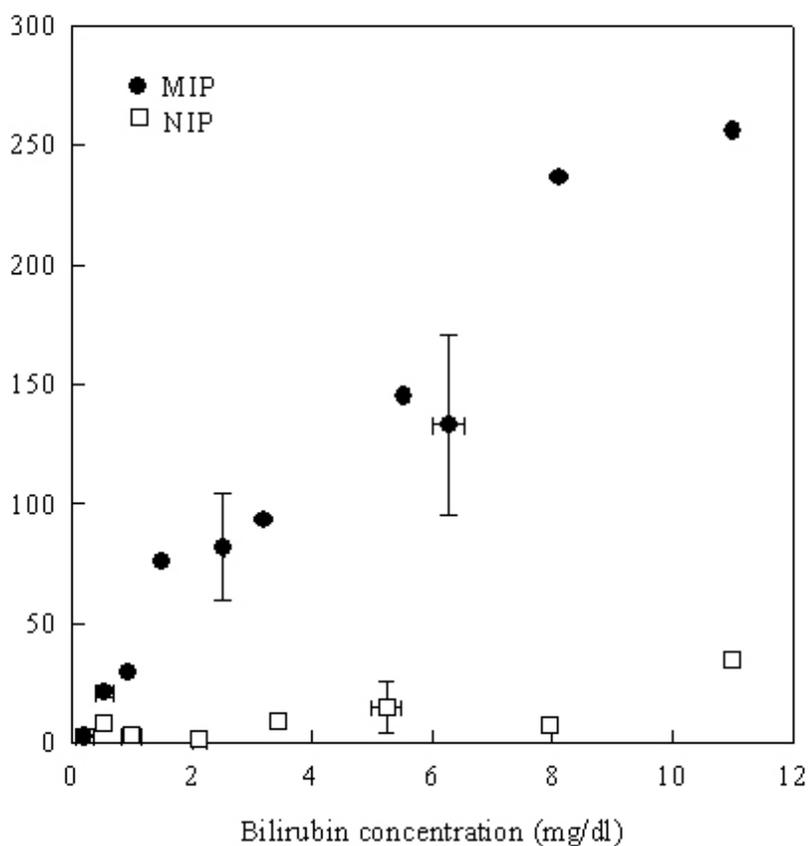


Figure 8 Comparison of QCM detection frequencies from the QCM chip coated with MIP and non-MIP films. The average RSD of the frequency shifts from MIP/QCM chip was 8.16%.

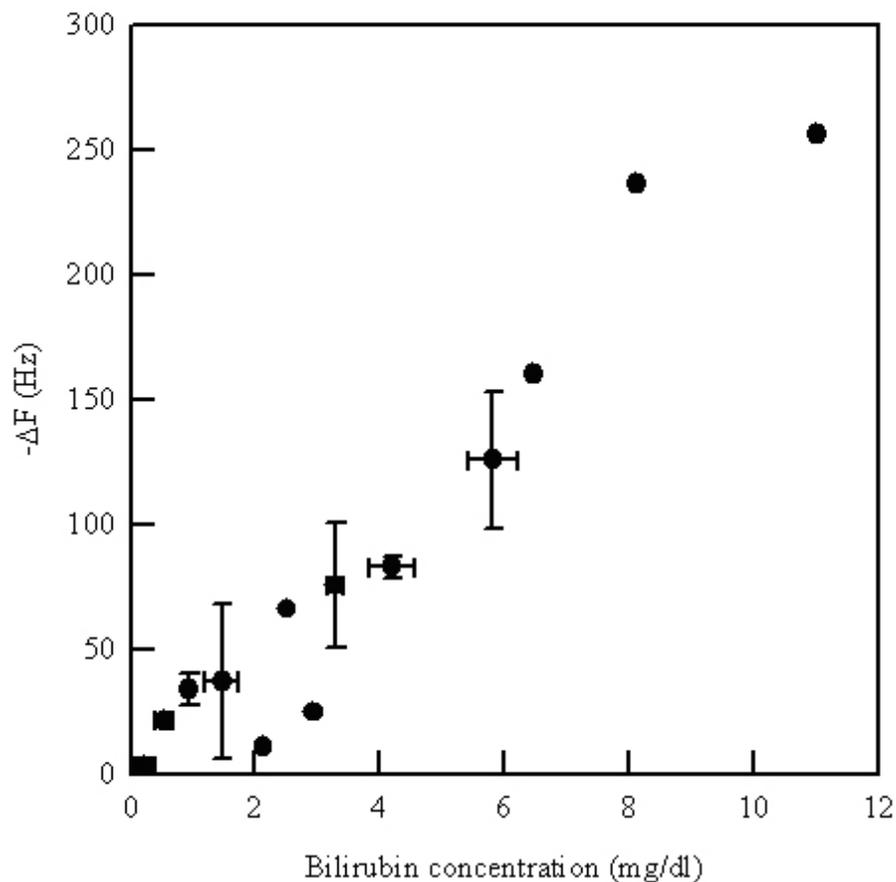


Figure 9 Reproducibility of frequency response from the same BIP/QCM chip. The average RSD was 15.84%.

The same BIP/QCM chip was used to test the reproducibility of the detection signal. After each injection of bilirubin and the attainment of equilibrium, the BIP/QCM chip was recovered by the injection of the solvent solution. In addition, the chip could be stored in deionized water at room temperature and reused for next experiments. The measurements from five different batches of operation are shown in Fig. 9. The average R.S.D. was 15.84%. The calibration correlations from these different batches were in good agreement. The slope for the linear calibration was 24.14 Hz/mg/dl with a correlation coefficient of as high as 0.913. Therefore, the detection signals were experimentally proved to be reproducible. Summarized from all the data used in this paper, it resulted in an average slope of 23.91 with an average R.S.D. of 0.9%. The slopes further confirmed the reproducibility and reliability of the detection. The BIP/QCM chip was found 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 chip has already proved its stability for more than seven months without loss of sensitivity, which is a significant advantage of the molecular imprinting technique.