

Picogram detection of metal ions by melanin-sensitized piezoelectric sensor

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Abstract

A heavy metal ion sensor was constructed by cross-linking melanin onto the gold electrode of quartz crystal microbalance (QCM). A mercury ion sensitivity of 518 ± 37 Hz/ppm was observed, a substantial increase in sensitivity compared to previous reports of 10–50 Hz/ppm with the limit of detection at 5 ppb. Detection of other metal ions including Sn^{2+} , Ge^{4+} , Li^+ , Zn^{2+} , Cu^{2+} , Bi^{3+} , Co^{2+} , Al^{3+} , Ni^{2+} , Ag^+ , and Fe^{3+} were also performed. Unexpectedly, binding of Mn^{7+} , Pb^{2+} , Cd^{2+} , and Cr^{3+} increased resonant frequencies. The surface profile of melanin thin film upon binding to metal ions was investigated by atomic force microscopy (AFM). Structural change of melanin upon binding to metal ions was characterized by circular dichroism and by infrared spectroscopy. The current study provides the first example of melanin-coated piezoelectric sensor showing high sensitivity and selectivity to metal ions.

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1. Introduction

A quartz crystal microbalance (QCM) is a mass-detection device that operates based on the piezoelectric properties of quartz crystals. The QCM is uncomplicated, cost-effective, real-time responding, high-resolution, and stable. Because of their extraordinary sensitivity and stability, QCMs have been applied in recent years as biosensors for the online detection of biomolecules (Cavic et al., 1999; Marx, 2003; Nakamura and Karube, 2003). However, there are still a very limited number of applications to the detection of heavy metal ions (Nomura and Iijima, 1981; Nomura and Sakai, 1986; Jane and Shih, 1995; Ng et al., 1998; Price et al., 2002; Casilli et al., 2004). Bipyridinium-coated electrode detects metal ions in the millimolar range (Casilli et al., 2004). Polythiophenes-functionalized QCM detects mercury and silver ions with a sensitivity of 10 Hz/ppm (Ng et al., 1998). Crown ether-coated electrode combined with a conductivity measurement detects metal ions at a

sensitivity of 50 Hz/ppm with a detection limit of 3×10^{-5} M (Jane and Shih, 1995).

Atomic adsorption spectroscopy (Hwang and Jiang, 1996), inductively coupled plasma optical emission spectroscopy (Jackson and Chen, 1996), inductively coupled plasma mass spectroscopy (Burlingame et al., 1996), high-performance liquid chromatography (Das-Ak Chakraborty et al., 1996), electrochemical separation (Hissner et al., 1996), and capillary electrophoresis (Wen and Cassidy, 1996) are commonly used in metal ion detection. For real-time detection, application of amperometric methodology detects nanomolar to femtomolar amounts of metal ions (Ugo et al., 1998; Bontidean et al., 1998). Although femtomolar detection has been achieved with various methods, application of QCM for the detection of heavy metal ions is still worth investigating due to its economy and convenience.

Melanin belongs to a category of phenolic polymers found in the black pigments of plants, animals, and microbes. Animal melanin is commonly divided into two types: black eumelanins and reddish-brown pheomelanins (Nicolaus, 1968; Crippa et al., 1989; Protá, 1998). The primary function of melanin is to protect the life form from the sun's direct radiation. In addition,

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melanin is (1) an antioxidant that possesses free radical scavenging activity, (2) a heavy metal chelator, and (3) an absorber of toxic organic species (Schwabe et al., 1989; Kollias et al., 1991; Liu et al., 1993; Smit et al., 2001; Hung et al., 2002a,b; Sava et al., 2002; Hung et al., 2003; Sava et al., 2003; Hung et al., 2004a,b; Izumi et al., 2005).

Recently, we extracted melanin from *Thea sinensis* Linn (Sava et al., 2001a,b). Tea melanin (TM) performs a wide range of biochemical and pharmacological roles in animals, including antioxidant, free radical scavenging, and in particular heavy metal ion binding activities (Hung et al., 2002a,b; Sava et al., 2002; Hung et al., 2003; Sava et al., 2003; Hung et al., 2004a,b). Applying melanin onto QCM electrodes might facilitate the development of heavy metal ion sensors (Hepel and Mahdavi, 1997; Hepel et al., 1997; Hepel, 1999; Diaz et al., 2005). The current study is based on the hypothesis that metal-binding might cause a dramatic change in the physical property of TM thin film, which might induce an additional frequency response of QCM (Sava et al., 2001a).

2. Experimental

2.1. Materials

Sephadex G-75 and molecular size markers were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals, which were of analytical grade or higher, were obtained from Merck (Darmstadt, Germany) or Sigma. Standard solutions of Hg^{2+} , Sn^{2+} , Ge^{4+} , Li^+ , Zn^{2+} , Cu^{2+} , Bi^{3+} , Co^{2+} , Al^{3+} , Ni^{2+} , Ag^+ , Fe^{3+} , Mn^{7+} , Pb^{2+} , Cd^{2+} , and Cr^{3+} were purchased from Sigma or Fluka and diluted to working concentration with Milli-Q ultra-pure water (18.2 M Ω cm).

2.2. Isolation of TM

TM was isolated using a procedure reported previously (Sava et al., 2001a,b), except that the extraction time was lowered to 12 h to avoid excessive oxidation of the TM. After extraction, the mixture was filtered and centrifuged at 15,000 \times g for 30 min to obtain the TM extract. This extract was acidified to pH 2.5 through the addition of 2N HCl and then centrifuged at 15,000 \times g for 15 min to form a pellet. Acid hydrolysis was employed to purify the TM. The purified product was dissolved in 0.2% NH_4OH and then precipitated. This precipitation procedure was repeated three times to sequester TM from any low-molecular-weight impurities and to improve its homogeneity. The preparation was treated with TiCl_3 , as described previously (Hung et al., 2002a,b) to fully reduce the functional groups and to improve the homogeneity of the monomers. The solutions were filtered through a Nalgene 0.45 μm syringe filter. Finally, TM was purified on a Sephadex G-75 column using 50 mM phosphate buffer (pH 7.5). The purified product was dissolved in 0.2% NH_4OH up to a concentration of 0.5%. A fraction of molecular weight 14 ± 3 kDa was isolated after purification through a Sephadex G-75 column; its purity, as examined by HPLC, was above 95%. Further study was performed using this purified fraction. Evidence from destructive

alkaline fusing, thin-layer chromatography (TLC), and mass spectrometry (MS) indicates that TM is composed mainly of flavonoids (Sava et al., 2001a,b). The apparent molecular weight of 14 kDa is equivalent to ca. 27 theaflavins in a melanic chain.

2.3. Immobilization of melanin on gold electrode and characterization by atomic force microscope (AFM)

One of the characteristic properties of melanic compounds is the strong binding affinity to metal ions. TM binds tightly to gadolinium ion (Hung et al., 2002a,b), however, binding against other metal ions is yet to be explored (Hepel and Mahdavi, 1997; Hepel et al., 1997; Hepel, 1999; Diaz et al., 2005). The fabricated quartz was immersed in 95% ethanol for 10 min, 1.2N NaOH for 20 min, Milli-Q water for 10 min, 1.2 M HCl for 5 min, Milli-Q water for 5 min, ethanol for 5 min, and air-dried. The gold electrode was reacted with 20 μL 2.5% glutaraldehyde at room temperature for 30 min, followed by Milli-Q water washing for 5 min. The activated electrode was reacted with 60 μL 0.5% TM for another 30 min, washed with Milli-Q water for 5 min, and allowed to air dry. A total of 5.3 ng TM (0.38 pmol) were coated onto gold electrode. AFM image was obtained using commercial DI5000 AFM (Nanoscope IIIa; Digital Instruments, Santa Barbara, CA) operating at tapping mode with a Si micro-cantilever. Images were scanned using scan rate 1 Hz and set point at 0.7 V.

2.4. QCM-FIA system

The oscillator (Catalog#35366-10) and flow-cell (Catalog#35363) were purchased from International Crystal Manufacturing Co. (Oklahoma City, USA). The QCM was fabricated from a 0.2 mm-thick AT-cut quartz wafer. The dimen-

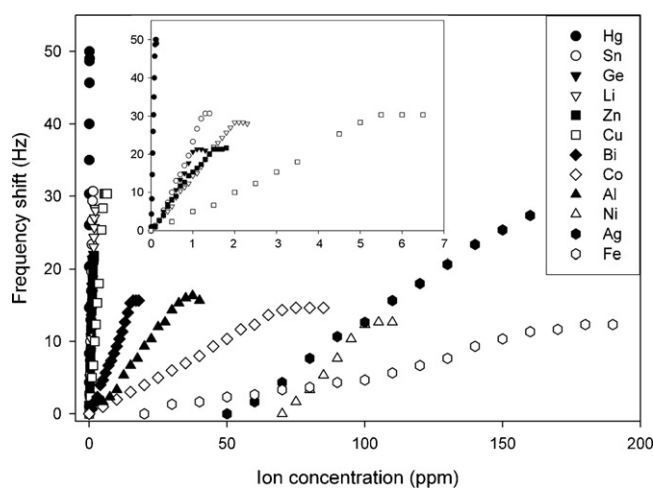


Fig. 1. Surveillance of metal ions by the QCM-FIA system. Metal ions at various concentrations are injected into QCM-FIA system, from which the corresponding frequency shifts are obtained. This plot contains all metal ions causing down-shift in resonant frequency, ions include Hg^{2+} , Sn^{2+} , Ge^{4+} , Li^+ , Zn^{2+} , Cu^{2+} , Bi^{3+} , Co^{2+} , Al^{3+} , Ni^{2+} , Ag^+ , and Fe^{3+} . The inset is a close-up magnifying a 0–6 ppm region containing Hg^{2+} , Sn^{2+} , Ge^{4+} , Li^+ , Zn^{2+} , and Cu^{2+} . Due to the ultra-high sensitivity, Hg^{2+} exhibits vertical lines even in the inset.

Table 1
Summary for binding reaction of metal ions to tea melanin-coated QCM

Ion	Compound	Sensitivity ^a (Hz/ppm)	Limit of detection ^b (ppm)	Saturation point ^c (ng)
Hg ²⁺	HgCl ₂	518 ± 37	0.005	0.55
Sn ²⁺	SnCl ₂	26 ± 7	0.1	6.5
Ge ⁴⁺	GeO ₂	21.4 ± 0.1	0.1	5
Li ⁺	Li ₂ CO ₃	15 ± 1.2	0.2	7.5
Zn ²⁺	Zn(NO ₃) ₂	14.42 ± 2.1	0.1	27.5
Cu ²⁺	Cu(NO ₃) ₂	5.3 ± 1.5	0.5	75
Bi ³⁺	Bi(NO ₃) ₃	1 ± 0.9	1.2	375
Co ²⁺	Co(NO ₃) ₂	0.2 ± 0.2	5.1	9.5
Al ³⁺	Al(NO ₃) ₃	0.52 ± 0.16	5.3	150
Ni ²⁺	Ni(NO ₃) ₂	0.43 ± 0.14	72.5	525
Ag ⁺	AgNO ₃	0.23 ± 0.24	65.8	850
Fe ³⁺	Fe ₂ O ₃	0.05 ± 0.03	30.8	900
Mn ⁷⁺	KMnO ₄	–2000	(1.8)	–
Pb ²⁺	Pb(NO ₃) ₂	–2500	(0.5)	–
Cd ²⁺	CdCl ₂	–348.9	0.02	–
Cr ³⁺	Cr(NO ₃) ₃	–1000	(0.4)	–

^a Sensitivity is defined as changes in frequency shift per ppm applied metal ion. Injection volume is 5 μL.

^b Limit of detection is defined as three times standard deviation of background noise. Numbers in parentheses are threshold concentrations for positively responding ions.

^c Saturation point is the upper limit of frequency shift plot.

sions of gold electrodes were 5 mm in diameter and 100 nm in thickness. A laboratory-constructed transistor–transistor logic integrated circuit (TTL-IC) was used to power the QCM. The TTL-IC was based on IC 74HC93, 74LS138, 74LS95, and 74LS04 (Kuan Hsi Co., Taiwan). An Agilent HP 53132 Uni-

versal Frequency Counter was used to monitor the frequency output. The melanin-coated QCM was assembled into a flow-cell unit. The final FIA system consisted of a cylindrical pump, injector, flow-cell, oscillation circuit, frequency counter, and personal computer (sFig. 1., supplemental information). The

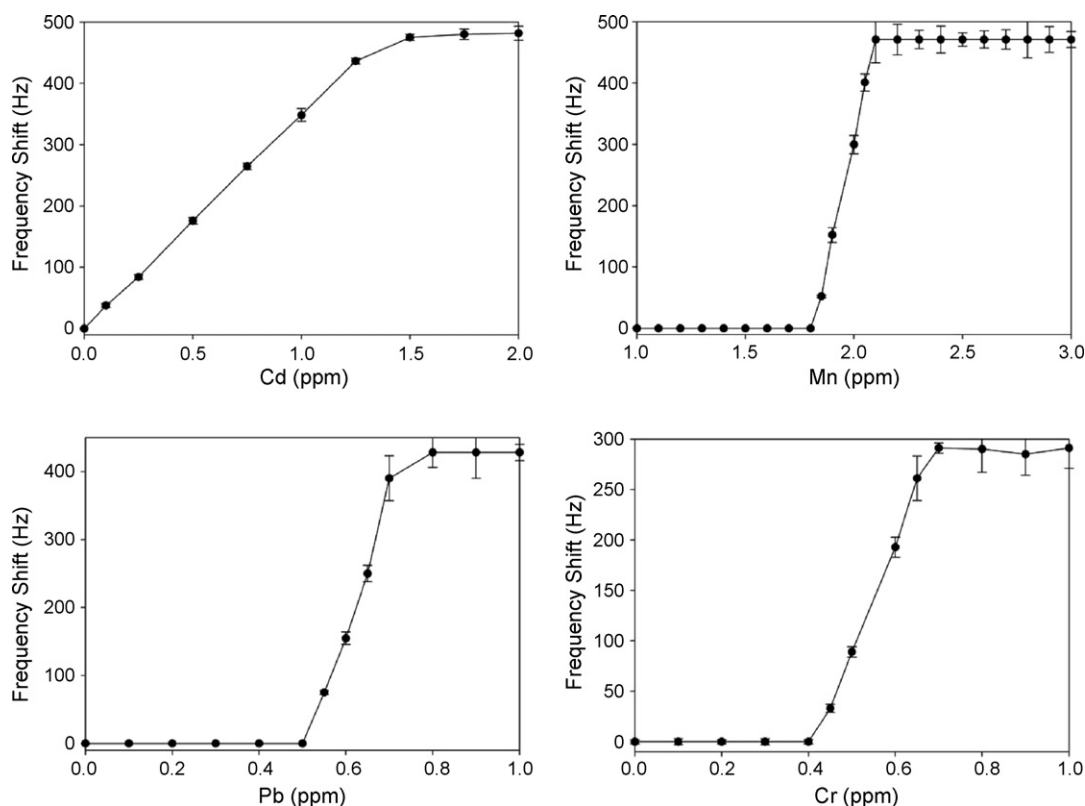


Fig. 2. Detection of Mn⁷⁺, Pb²⁺, Cd²⁺, and Cr³⁺ by the QCM-FIA system. Concentration vs. frequency shift is drawn for Mn⁷⁺, Pb²⁺, Cd²⁺, and Cr³⁺. Binding of these ions up-shifts QCM resonant frequency, which is atypical for QCM detection. Cd²⁺ shows slope of 350 Hz/ppm. Others exhibit thresholds of ca. 0.4–1.8 ppm and with steeper drops in frequency shift. Relative standard deviation (R.S.D.) is shown as error band in each curve.

fundamental resonant frequency for the QCM-FIA system was 9.995 MHz. All analyses were carried out using Milli-Q ultra-pure water (18.2 M Ω cm) and solutions of analytical grade at 25 °C. The QCM-FIA system was pre-run at 3 mL/h for 30 min. A volume of 5 μ L was applied for each injection. The TM-coated electrode was regenerated by 50 μ L injection of 0.1N HCl followed by 10 min Milli-Q water wash. Repeated regeneration caused a decrease in binding capacity but did not affect sensitivity. The TM-coated electrode was regenerated five times for optimal detection. The crystal was repeatedly coated, cleaned, and washed until the gold electrode fell off.

3. Results

3.1. Detection of metal ions by the QCM-FIA system

Five microliters of metal ions at various concentrations were injected into the QCM-FIA system and monitored by resonant frequency in real time. Detection of metal ions including Hg²⁺, Sn²⁺, Ge⁴⁺, Li⁺, Zn²⁺, Cu²⁺, Bi³⁺, Co²⁺, Al³⁺, Ni²⁺, Ag⁺, and Fe³⁺ was performed. Binding of metal ions onto bare electrode and dextran-coated electrode was performed in parallel as control experiments. For both control experiments, no frequency drop was observed up to 10 ppm Hg²⁺. Binding of metal ions onto the TM-coated electrodes decreased the resonant frequency. The negative frequency shift per applied weight, defined as sensitivity, remained constant at initial metal ion concentrations and reached a plateau at higher concentrations. The sensitivity, limit of detection (LOD), and saturation point were obtained from Fig. 1 and listed in Table 1. In particular, mercury ion exhibited distinct sensitivity of 518 \pm 37 Hz/ppm with the limit of detection (LOD) 0.005 ppm and the saturation point at 0.55 ng. Sn²⁺, Ge⁴⁺, Li⁺, Zn²⁺, and Cu²⁺ exhibited (medium-strength) binding affinity with the sensitivity ranging from 5.3 \pm 1.5 Hz/ppm to 26 \pm 7 Hz/ppm while Bi³⁺, Co²⁺, Al³⁺, Ni²⁺, Ag⁺, and Fe³⁺ showed much lower sensitivity (Table 1). In summary, melanin-coated electrodes are capable of selectively binding various metal ions.

Unexpectedly, when a group of ions including Mn⁷⁺, Pb²⁺, Cd²⁺, and Cr³⁺ was injected into the QCM-FIA system, a raise in resonance frequency was observed (Fig. 2). This group of ions showed high sensitivities ranging from 350 Hz/ppm to 2500 Hz/ppm (Table 1). The frequency shift plots of Mn⁷⁺, Pb²⁺, and Cr³⁺ showed threshold concentrations (0.4–1.8 ppm). For concentrations higher than threshold concentrations there is a narrow linear responding zone (ca. 0.3 ppm) before reaching a plateau.

4. Discussion

It is possible that there exists more than one type of binding site on melanin. To clarify this conjecture, the melanin-coated gold electrode was saturated with 0.2 ppm mercury ion followed by injection of 5 ppm Sn²⁺, 5 ppm Ge⁴⁺, or 5 ppm Zn²⁺ (Fig. 3). No additional frequency shift was observed when metal ions were injected into QCM pre-saturated with mercury. This evi-

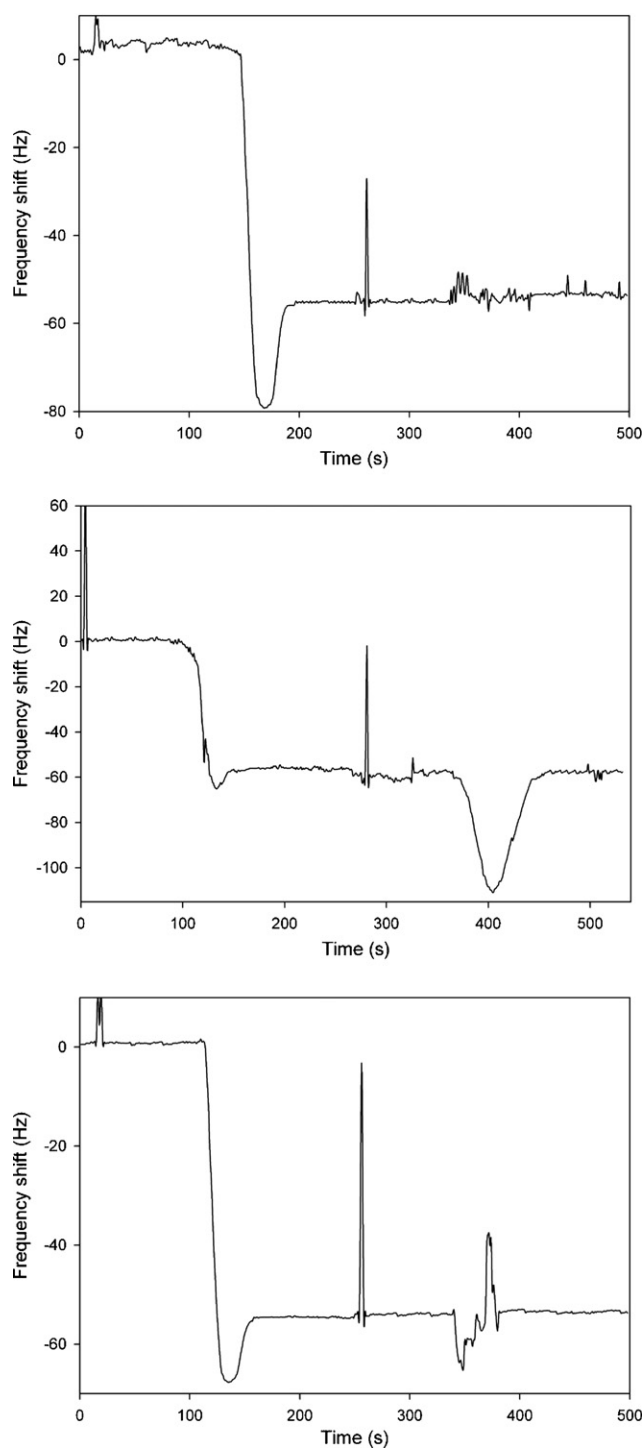


Fig. 3. Real-time measurement for the competition binding of Sn²⁺, Ge⁴⁺ and Zn²⁺ to melanin-coated gold electrode pre-saturated with Hg²⁺. The QCM-FIA system is pre-saturated by injection of 0.2 ppm mercury ion followed by injection of 5 ppm Sn²⁺ (top), 5 ppm Ge⁴⁺ (middle), or 5 ppm Zn²⁺ (bottom). The injection peaks for various ions are indicated on the plots. No difference in the resonant frequency indicates that all available binding sites for Sn²⁺, Ge⁴⁺ and Zn²⁺ are occupied by pre-injected mercury ions.

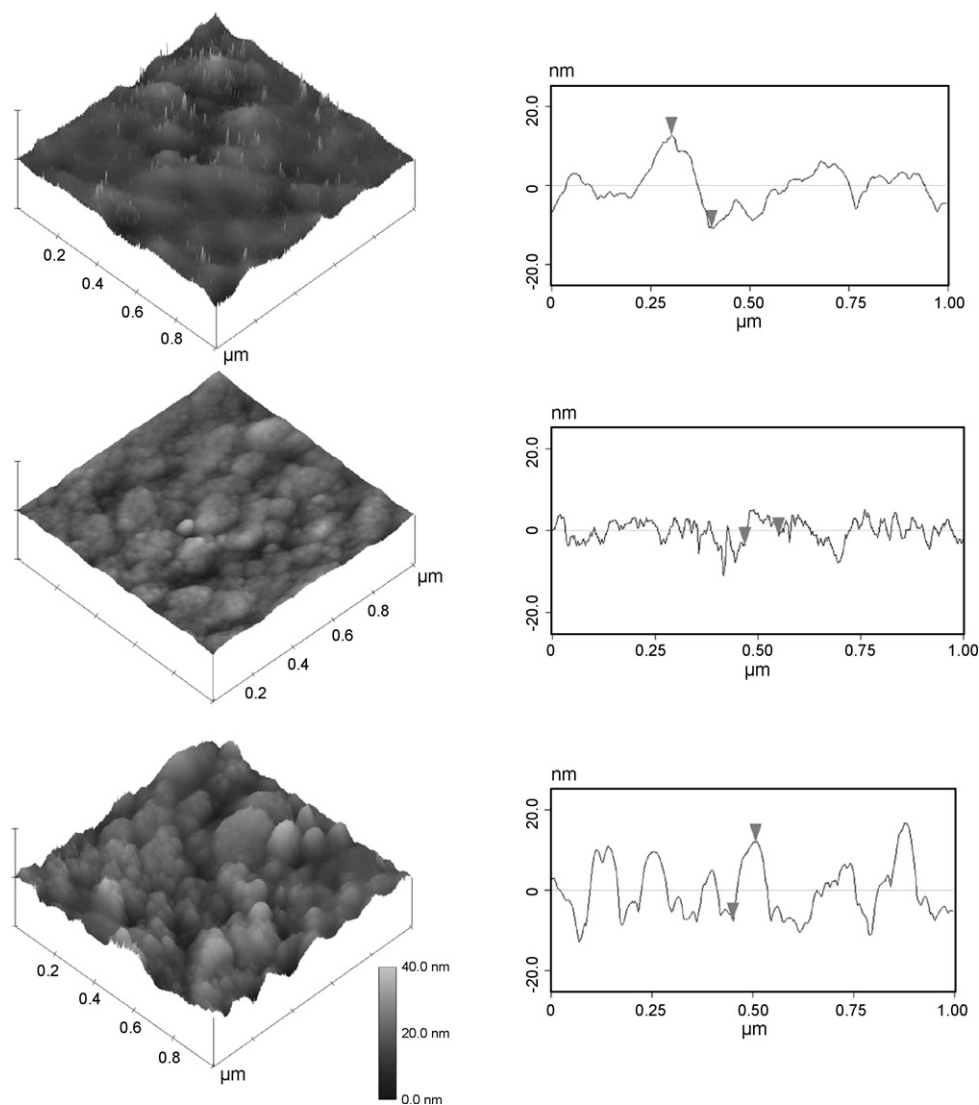


Fig. 4. Images obtained from atomic force microscopy for the characterization of surface profiles on melanin-coated gold electrodes. One μm by one μm area is scanned by AFM using tapping mode. Surface profiles of gold electrode before treatment (top image), after melanin coating (middle image), and after mercury ion binding (bottom image) were obtained. A cross-section of each sample is shown on the right. The color code for depth is shown at bottom right.

dence strongly suggested that melanin contains only one type of metal ion binding site and the observed differences in the sensitivities are solely due to the differences in the binding affinity of metal ions to the same binding site.

Binding stoichiometry was obtained by calculating the molar quantity of mercury ion needed to saturate the TM-coated electrode and divided by coated TM molecules. The stoichiometry of Hg^{2+} versus TM was 1:3 (0.13 pmol Hg^{2+} versus 0.38 pmol TM). It is possible that due to the random orientation of TM cross-linked onto electrode, a large fraction of metal-binding sites might be buried or destroyed by glutaraldehyde. In reality, we could assume a 1:1 binding stoichiometry.

The LOD of mercury ion within a QCM-FIA system in the current study (5 ppb) approached the detection limit generally quoted for a QCM system in gas detection, which is much more sensitive than most QCM detection in liquid flow-cell system (Jane and Shih, 1995; Ng et al., 1998; Casilli et al., 2004). In addition to mass-induced frequency shift, this sensitivity might

come from the ion-induced alternation of surface property. To explore possible morphological change, the surface profiles of naked electrode, melanin-coated electrode, and Hg^{2+} -saturated melanin-coated electrode were examined by atomic force microscope at tapping mode (Fig. 4). Cross-section showed that the general appearance of the unmodified electrode was smooth with gold polycrystalline hills 200 nm wide. Coating of TM created bumps ca. 50–100 nm wide and 10 nm high. Mercury ion binding significantly enhanced the roughness, but the lateral dimension of bumps remained unchanged. AFM image indicated that ion binding induced a dramatic change in the surface profile.

To obtain possible conformational change of melanin upon binding to metal ions, circular dichroism (CD) was performed to monitor the reaction of melanin binding to Hg^{2+} and Co^{2+} (Fig. 5). A decrease of the CD signal at 220 nm was observed. Infrared (IR) spectroscopy of the TM thin film was also performed to show structural characteristics upon binding to metal ions (Fig. 5). Tea melanin showed multiple peaks from

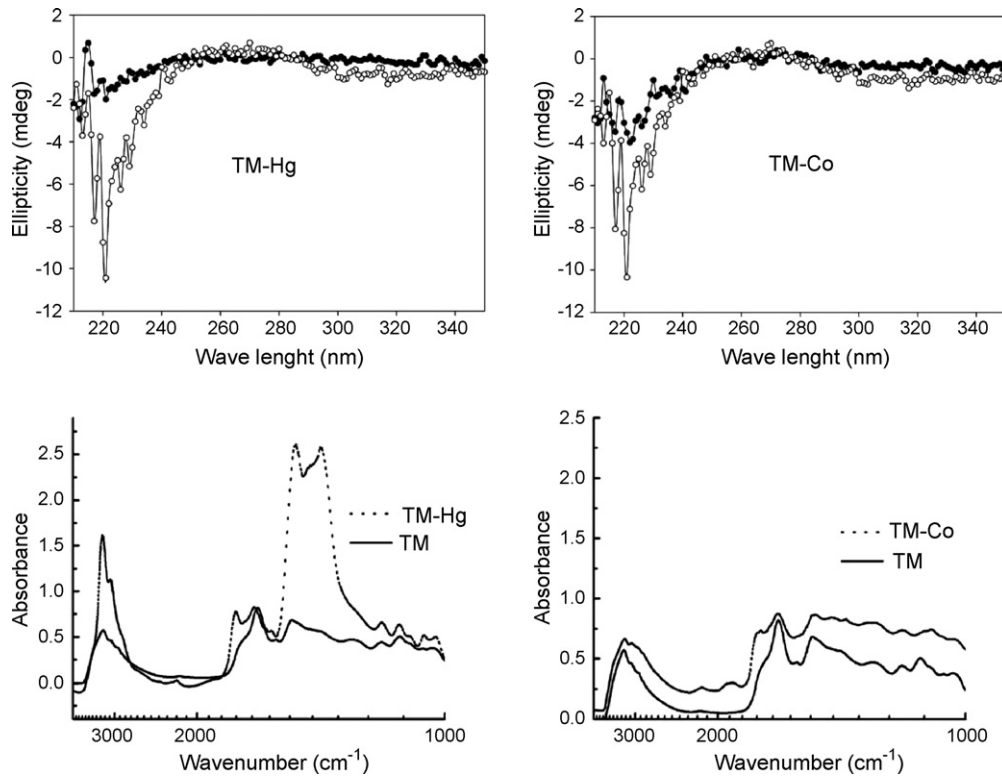


Fig. 5. CD and IR spectra of TM and TM-metal thin film. Melanin–metal ion complex was derived by adding metal ions to 1 mg/mL melanin solution at final concentration of 1 ppm. CD spectra of metal ions, melanin, and melanin–metal ion complex were recorded. CD spectra of metal ions were added to melanin spectrum (open circles) and compared to CD spectrum of melanin–ion complex (filled circles). Incorporated metal ions are Hg^{2+} (left) and Co^{2+} (right). To obtain IR spectra, melanin or melanin–metal ion complex were deposited onto silicon wafer to generate thin films. Shown at the bottom are IR spectra, which indicate a significant difference for metal-bound melanin (dashed line) vs. TM thin film (solid line). Incorporation of Hg^{2+} (left) and Co^{2+} (right) are shown.

1200 cm^{-1} to 1720 cm^{-1} and a broad band at 3450 cm^{-1} . The presence of wide band at 3450 cm^{-1} attributed to stretching vibrations of OH and NH_2 groups, typical for melanin. The strong absorbance at 1720 cm^{-1} was assigned to stretching vibrations of free carboxylic groups. A strong absorption at 1650 cm^{-1} was recognized as the vibration of aromatic C=C and C=O groups. In the presence of mercury ion we observed strong absorption at 1370 cm^{-1} and 1480 cm^{-1} , which indicated scissoring and bending of CH group. An increase of absorbance for the broad band at the 3450 cm^{-1} was also observed. Binding of mercury ion induced strong absorbance of these two peaks while binding of cobalt ion caused minimal response. Evidence from CD and IR spectra indicated that melanin underwent structural rearrangement upon binding to metal ions. The picogram quantity of mercury ion when bound to melanin thin film was capable of inducing global changes in its physical property which was reflected by the dramatic shift in the resonant frequency. Thus, the frequency shift of melanin-coated QCM is at least due to the mass-change and structure-induced frequency response.

The mechanism causing positive frequency response induced by the binding of Mn^{7+} , Pb^{2+} , Cd^{2+} , and Cr^{3+} is not clear. It is possible that the binding induced conformational change, which modified the damping property of QCM and promoted resonant frequency in a linear fashion. Alternatively, chemical reaction might have been induced by the metal binding which might

have released energy and increased the apparent resonant frequency. The sigmoidal frequency response implied a two-state phase transition between two stable states (Fig. 3). However, this phenomenon requires further study regarding structural and physical characterization as to the stable states and transition state.

5. Conclusions

The current study is the first time application of melanin-coated QCM as a metal ion detector used to study the binding affinity of metal ions against melanin. We have constructed a piezoelectric sensor system capable of real-time detection of metal ions. The major component is a melanin-coated QCM embedded in the flow-cell of an FIA system. The resulting system exhibits sensitive detection for Hg^{2+} . Binding of Mn^{7+} , Pb^{2+} , Cd^{2+} , and Cr^{3+} promoted resonant frequency. The current study provides the application of QCM as a convenient platform to study the binding properties of melanin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bios.2007.04.011](https://doi.org/10.1016/j.bios.2007.04.011).

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