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Detection of cadmium by a fiber-optic biosensor based on localized surface plasmon resonance

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ABSTRACT

A novel transmission-based localized surface plasmon resonance (LSPR) fiber-optic probe has been developed to determine the heavy metal cadmium ion (Cd(II)) concentration. The LSPR sensor was constructed by immobilizing phytochelatins (PCs), (γGlc-Cys)$_8$-Gly, onto gold nanoparticle-modified optical fiber (NM_{Au}OF). The optimal immobilizing conditions of PCs on to the NM_{Au}OF are 71.6 μg/ml PCs in pH 7.4 PBS for 2 h. The absorbability (change of light absorption) of the PC-functionalized NM_{Au}OF sensor increases to 9% upon changing the Cd(II) level from 1 to 8 ppb with a sensitivity of 1.24 ppb^{-1} and a detection limit of 0.16 ppb. The sensor retained 85% of its original activity after nine cycles of deactivation and reactivations. In addition, the sensor retains its activity and gives reproducible results after storage in 5% D-(+)-trehalose dehydrate solution at 4 °C for 35 days. The dissociation constant (K_d) of the immobilized PCs with Cd(II) was about 6.77×10^{-8} M. In conclusion, the PCs-functionalized NM_{Au}OF sensor can be used to determine the concentration of Cd(II) with high sensitivity.

1. Introduction

Detection and quantitation of toxic heavy metals is an important issue with the increasing environmental concern about drinking water safety. Traditionally, atomic absorption (AA) spectroscopy, inductively coupled plasma (ICP) optical emission spectrometry, and inductively coupled plasma mass spectrometry (ICPMS) are widely used methods for the determination of heavy metals (Anderson et al., 1996; Burlingame et al., 1996; Jackson and Chen, 1996). These methods are sensitive and allow for the discrimination of different metal ions but require tedious sample pretreatments (e.g., desalting, filtration and concentration). Therefore, it is essential to develop a rapid and sensitive screening method to ascertain the presence of metal ions for in situ environmental monitoring.

A lot of efforts have been devoted to develop inexpensive enzymatic or peptide methods for the detection of heavy metals for industrial processes and environmental monitoring. The enzyme or peptide may be immobilized on a suitable surface or supporter, and its binding or inhibition can be measured by different detection techniques such as ion-sensitive field effect transistors, potentiometric and piezoelectric devices, amperometric electrodes, and optical-based biosensors (Bontidean et al., 1998, 2003; Saber and Piskin, 2003; Lee and Russell, 2003; Blake et al., 1998). In this study, cadmium ion (Cd(II)) has been chosen as the target analyte, since it is representative of a ubiquitous environmental pollutant. Phytochelatins (PCs) as the heavy metal ion binding peptides consist of L-glutamic acid, L-cysteine and a carboxy-terminal glycine. These compounds, occurring in plants and some fungi with the general structure (γ-Glu-Cys)$_n$-Gly (n = 2–11), are metallothionein-like substances (Grill et al., 1985, 1986; Rauser, 1995). They are capable of chelating Cd(II) by thiolate coordination because of induction by the addition of Cd(II), high capacities of Cd-binding, and a high content of Cys-residues (Kondo et al., 1984). Since it is tedious and time consuming to isolate and purify PCs from plants or organisms, PCs can be massively produced up to 26 mg/l by using recombinant DNA and fermentation technologies.

Recently, a novel fiber-based biosensor based on the particular optic properties of gold nanoparticles has been developed (Lin et al., 2006; Chau et al., 2006). When an optical field is incident upon noble metal nanoparticles, absorption occurs if the optical frequency is resonant with the collective oscillation of the conduction electrons, which is known as the localized surface plasmon resonance (LSPR), which is absent in the bulk metal. The local environment of the metal nanoparticles has a significant influence on the resonance frequency and absorption of LSPR (Jensen et al., 1999; Nath and Chilkoti, 2002). The light absorption level by gold nanoparticles is sensitive to the refractive index (RI) of the surrounding solvent and, in addition, to the binding events of those functionalized nanoparticles. With a suitable receptor

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immobilized at the surface of the gold nanoparticles, the resulting LSPR fiber-optic sensor can detect the corresponding analyte even if there is no emission and no absorption in the UV–vis region. Therefore, the presence of analytes can be determined directly without the use of labeled molecules (Lin et al., 2006; Nath and Chilkoti, 2002).

For this study, a PCs-functionalized fiber-based biosensor utilizing the LSPR effect was developed to evaluate the concentration of Cd(II). First, a layer of the gold nanoparticle was immobilized on the outside of the fiber to couple with the evanescent wave. By using a self-assembling technique, a bioactive layer consisting of genetically synthesized PCs was immobilized by covalent coupling onto the gold nanoparticle layer and the optimal conditions of immobilization were examined. When the PCs binding with Cd(II) takes places, the local refractive index was altered and hence the transmission. Based on the correlation between binding rate and light attenuation, the concentration of Cd(II) can be determined. Several factors, including reactive rate, stability, and binding constant, were investigated as well.

2. Materials and methods

2.1. Reagents and materials

Multimode plastic-clad silica optical fiber (0.37 NA, model F-MBC) was purchased from Newport (Irvine, CA) with core and cladding diameters of 400 and 430 μm, respectively. Phytochelatins ($(\gamma$Glc-Cys$)_8$-Gly) were provided by the Development Center for Biotechnology, Taipei, Taiwan. The following chemicals, *n*-hexadecyltrimethylammonium bromide (CTAB, Fluka), sodium borohydride (Lancaster), 3-(mercaptopropyl)-trimethoxysilane (MPTMS, Acros), cystamine dihydrochloride (Sigma), phosphate buffered saline (PBS, Sigma), *N*-(3-dimethylaminopropy)-*N*-ethylcarbodiimide hydrochloride (EDC, Sigma), dithiothreitol (DTT, Sigma), *N*-hydroxysuccinimide (NHS, Sigma), D-(+)-trehalose dehydrate (Sigma), were used as received. All aqueous solutions were prepared with water purified using YMDI-100 water purification unit (Yeameei Membrane) with a specific resistance of 18 MΩ cm.

2.2. Production of PCs

The cells of *Escherichia coli* (*E. coli*) Top10 and BL21x transfected by vectors pCR2.1-TOPO and pTrcHis containing the PCs were cultured at 37 °C in 1.5 l of 2YT medium (10 g/l yeast extract, 16 g/l tryptone and 5 g/l NaCl) with ampicillin (100 μg/ml). When the absorbance (OD_{600}) reached 0.5, 2.5 ml IPTG (isopropyl-β-D-thiogalactopyranoside) (100 μg/ml) was added into the culture medium. After 16 h of protein induction, 20 μg/ml Cd(II) was added after an overnight growth period in 2YT medium. Then, the cells were lysed to break the cell walls using lysozyme (1 mg/ml) and ultrasonic probe. The lysed cells were centrifuged at 4000 × *g* at 4 °C for 30 min to obtain a clarified supernatant for immobilized metal-chelated affinity chromatography. The desired peptide (i.e., PCs) was isolated by Ni-NTA column metal-chelated affinity chromatography techniques. The purity of the peptide was monitored by sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-Protean II apparatus and a Tris-glycine buffer system was used to monitor purification during chromatography. The gels were run under reducing conditions with heat treatment of the samples (95 °C, 5 min) and electrophoresis was performed for 45 min at 200 V using 12% polyacrylamide gels. Detection was performed with Coomassie brilliant blue R250 staining.

2.3. Preparation of gold nanoparticle-modified optical fiber

First, colloidal Au solution was prepared by mixing hydrogen tetrachloraurate (1.78 ml, 25.4 nM), 8.22 ml of chloroform, and 0.4 ml of a 0.02 M ethanol solution of CTAB for 10 min to form a 4.52×10^{-4} M hydrogen tetrachloroaurate solution. Freshly prepared $NaBH_4$ ethanol solution (0.8 ml, 0.15 M) was added to the hydrogen tetrachloroaurate solution with vigorous stirring for 30 min. Histograms derived from TEM image analysis showed that the mean diameter of Au nanoparticles was 9.6 ± 2.3 nm. Then, the unclad portion (5 cm) of the optical fibers was cleaned for 30 min in a bath consisting of 3 vol of 30% H_2O_2 and 7 vol of concentrated H_2SO_4. The clean unclad portions of the optical fibers were then submerged into vials of 1% solution of MPTMS in toluene. After 8 h, the optical fibers were rinsed with methanol to remove unbound monomers from the surface. After thorough rinsing, the unclad portions of the optical fibers were immersed in Au solution for 5 h to form a self-assembled gold nanoparticle monolayer (NM_{Au}) on the core surface. Subsequently, the modified optical fibers were rinsed sequentially with water, methanol, and chloroform. The AFM surface topography of the prepared gold nanoparticle-modified optical fiber is shown in Fig. 1(a).

2.4. Immobilization of PCs onto NM_{Au}

First, NM_{Au} was modified to form a self-assembled monolayer (SAM) of cystamine by immersing NM_{Au} in 0.02 M cystamine dihydrochloride (pH 7.4 in PBS) for 2 h to form an amine functional group. The cystamine-modified NM_{Au} was further immersed in a PBS solution containing 450 mM EDC, 90 mM NHS, and various concentrations of PCs for 2 h at room temperature, rinsed with PBS, and air-dried at room temperature. Then, the amine group of cystamine modified on the NM_{Au} can couple with the activated succinimide esters reacting from PC and NHS, and finally form an amino bond between cystamine and PC, as shown in Fig. 1(b). The concentration of PCs, pH of the solution, and incubation time were varied to find the optimal immobilization conditions. Prior to the following tests, the PCs-immobilized LSPR sensors were activated by 2% DTT for 20 min, and rinsed with DI water. Fig. 1(a) shows an AFM surface topography of PCs-modified NM_{Au} probe. The 4-μm^2 image contained a random distribution of PCs on the surface with 13 nm roughness. The morphology clearly indicates that the PCs immobilizing treatments were successfully performed on the surface of NM_{Au}.

2.5. Cd(II) detection by PCs-modified sensors

In the LSPR sensors, the light attenuation will be affected upon capturing or reaction of analyte molecules by a molecular recognition element immobilized on the sensor surface. For this case, the reaction was carried out in a standard solution. When Cd(II) binds with PCs, the decrease in light intensity is correlated to the concentration of Cd(II). The degree of absorbability can be calculated according to the following formula:

$$\text{absorbability}\,(\%) = \frac{I_0 - I_1}{I_0} \times 100 \quad (1)$$

where I_0 and I_1 are the average light intensity measured at the initial PCs activity without and with Cd(II), respectively. According to World Health Organization (WHO), the Cd(II) concentration in drinking water should be less than 5 ppb. In this study, the detection range of Cd(II) is chosen from 0 to 8 ppb in 0.02 M Tris buffer solution (pH 6.5).

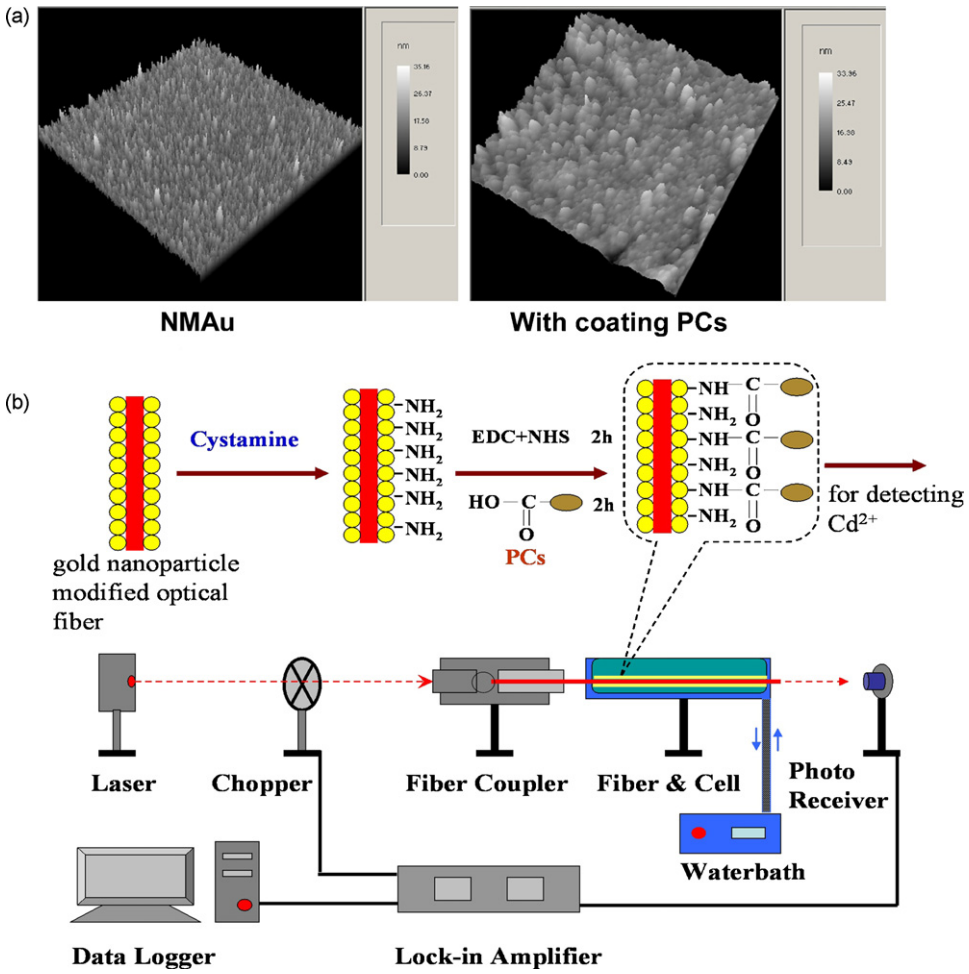

Fig. 1. (a) AFM surface topographies of the NM_{Au} and PCs-modified optical fiber; (b) the LSPR biosensor system and the chemical reactions scheme carried out for covalent binding of PCs to the fiber-based LSPR sensor.

2.6. Reactivation and stability of PCs-modified sensors

To test whether the sensor can be reactivated after single bolus treatment with high concentration of Cd(II), the buffer solution (0.1 M sodium acetate and 1 M $CaCl_2$, pH 3.6) is used to reactivate the PCs right after the sensor is deactivated by a 500 ppb Cd(II) for 10 min. Following the reactivation by the buffer solution for 2 h, the re-activated sensor is again immersed in the Cd(II) solution to measure the light intensity and to check the change of calibration curve. For evaluating the stability, the sensor was immersed in a 5% D-(+)-trehalose dehydrate solution and was stored in a 4 °C refrigerator. The preserved sensor was taken out for Cd(II) detection test and reactivated by the buffer solution after 7, 14, 21, 28, and 35 days, respectively.

2.7. Instrumentation and measurements

The optical configuration for the gold nanoparticle-modified optical fiber biosensor is a transmission-based fiber-optic type, as shown in Fig. 1(b). Basically, the system consists of a laser (Hitachi HL6320G laser diode, 635 nm, 10 mW; Thorlabs LDC500 laser diode controller; Thorlabs TEC2000 temperature controller; Thorlabs TCLDM9 laser mount), a chopper (Stanford Research SR540), a fiber coupler, a beam splitter, a sensing fiber, a liquid cell (10 ml), a photo-receiver (Thorlabs PDA55), and a lock-in amplifier (Stanford Research SR830). For the detailed description of the biosensor system, please refer to Chau et al. (2006). The temperature of the liquid testing cell was maintained by a circulatory waterbath (Wisdom, model LC-06) within ±0.05 K. By a pipette, the Cd(II) solution was gently filled into the liquid testing cell to immerse the LSPR sensor, which was installed horizontally in the cell. After reaching a balance, a new stable response can be attained in 5 min. However, to make sure that reaction reaches to an equilibrium state, the responding signals are taken over 10 min after the addition of Cd(II) solution. In this work, at least three replicates were performed for each parameter.

3. Results and discussion

3.1. Comparing probe with/without immobilizing PCs on NM_{Au}

While applying the LSPR sensor, the capture of Cd(II) by the immobilized PCs causes a decrease in light intensity at the detector due to an increase in the local RI. Fig. 2 shows an example of serial responding signals from the PCs-modified LSPR sensor immersed in a 0.02 M Tris buffer solution after sequential addition of Cd(II) to final concentrations of 8 ppb. The responding signals decrease with increasing Cd(II) concentration at room temperature and with a good linearity (which will be discussed in the later section). Notably, under the same operating conditions, there is no significant response change for the fiber without immobilizing PCs, suggesting that the genetically synthesized

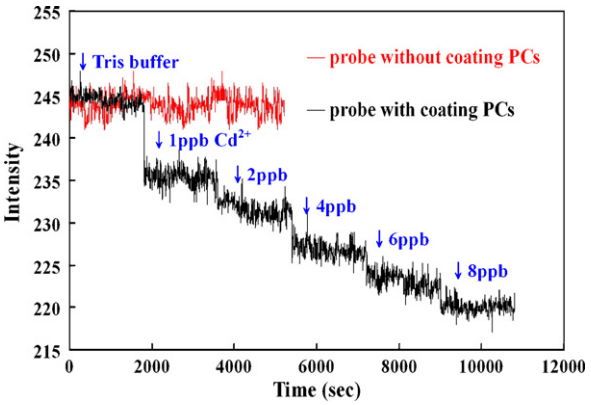

Fig. 2. An example of serial Cd(II) response signal in the range of 1–8 ppb by two sensors with/without PCs coating.

PCs can substantially differentiate the change in Cd(II) at low concentrations.

3.2. Optimal PCs immobilizing conditions

The sensitivity of an optic-fiber sensor is related to the immobilizing conditions, such as the amount of immobilized ligand and pH value of the solution (Jorgenson and Yee, 1993). The amount of immobilized ligand is determined by the ligand concentration and incubation period. Fig. 3 shows the effects of PCs concentration, incubation time, and pH on the response signals for Cd(II), respectively. In theory, higher incubating PCs concentration (or longer incubating time) revealed larger immobilization of PCs onto the fiber, resulting in a stronger LSPR effect and a smaller I_0. Besides, the sensor immobilized with more PCs has the higher residues to react Cd(II) with a smaller I_1, results in a higher absorbability and resolution with a rather good linearity. From Fig. 3(a) and (b), it can be seen that as the incubating PCs concentration and time increases from 35.8 μg/ml and 1 h, respectively, to 71.6 μg/ml and 2 h, the absorbability reaches a maximal value of 11.5% with a good linearity (R^2 = 0.996) for Cd(II) concentration ranging from 1 to 8 ppb. However, upon further increase in the incubating enzyme concentration or time, the amount of immobilized PCs onto the fiber becomes over saturated and causes steric hindrance or denaturation from binding with Cd(II), leading to a nonlinear response in absorbability.

Fig. 3(c) shows the plots of the absorbability versus Cd(II) concentration with different sensors incubated in various pH values of 71.6 μg/ml PCs solution for 2 h. The response is better (i.e., larger absorbability change and better linearity) for the sensor incubated with PCs in basic conditions than in acidic conditions. In basic conditions, the maximal absorbability change is found at pH 7.4, after which the absorbability decreases. The two possibilities are: in the lower pH, the coupling between succinimide esters and amine group of cystamine is less effective to form the amino covalent binding (Gestwicki et al., 2002). On the other hand, the peptide denaturation of PCs under too low or too high pH immobilization may contribute to its lower activity. According to previous studies by Yan et al. (2000), the activity of PCs is more sensitive toward the alkaline condition than the acidic condition and has an optimal pH of around 7.5, which is matched to our finding of 7.4. In summary, the optimal immobilization conditions for modifying the LSPR sensor are 71.6 μg/ml PCs and pH 7.4 for 2 h incubation, and will be applied for the rest of the studies. With the limit of detection (LOD) defined as the intensity at a Cd(II) concentration that yields a signal-to-noise (S/N) ratio of 3, the LOD of the sensor with optimal immobilization is 0.16 ppb. Besides, with the sensitivity defined as the ratio of the change in absorbability to an increase in a detecting concentration, the sensitivity of the sensor with optimal immobilization is 1.24 ppb^{-1}.

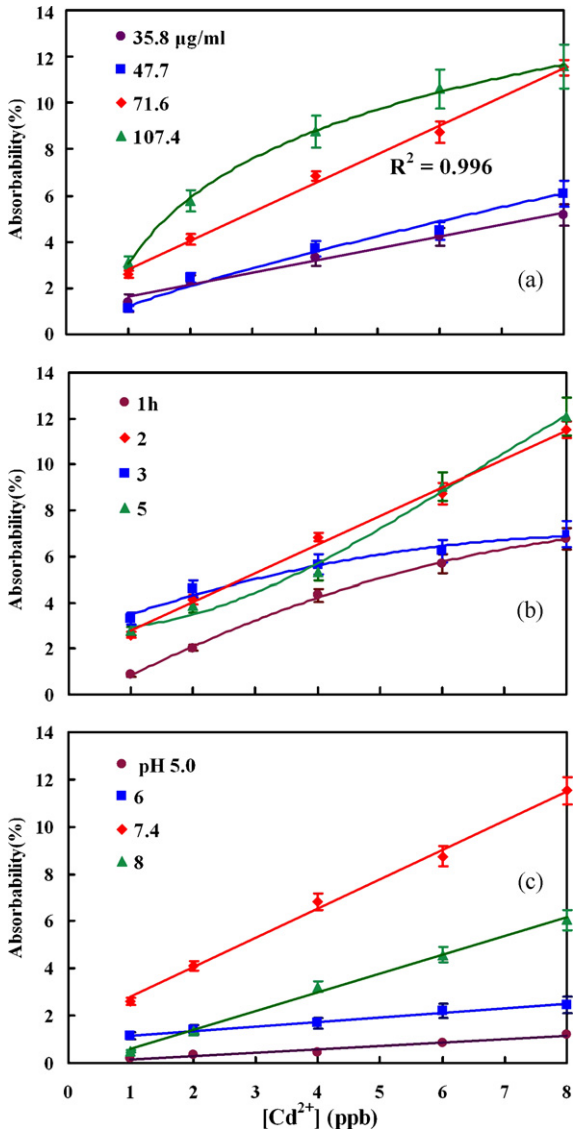

Fig. 3. Effects of (a) the incubating PCs concentration, (b) incubating time and (c) pH on the response for detecting 1–8 ppb Cd(II) in pH 6.5 and 0.02 M Tris buffer solution at 25 °C.

3.3. Effects of temperature and other metal ions on the sensor

PCs are short cysteine-rich peptides, of which activity depends on temperature. Hence, the PC binding capability with heavy metal ions is strongly affected by temperature (Yan et al., 2000). Fig. 4 shows the response of absorbability for detecting 1–8 ppb Cd(II) under different detecting temperatures. From this figure, the optimal detecting temperature for PCs responding to Cd(II) ranges from 25 to 41 °C, after that the change of absorbability becomes less. Considering the response linearity, 25 °C with R^2 = 0.996 was chosen as the detecting temperature for the PCs-functionalized LSPR sensor.

Another important issue on whether the PCs-functionalized LSPR sensor is able to bind to other metals needs to be addressed. Besides Cd(II), the bindings of magnesium (Mg(II)), copper (Cu(II)), lead (Pd(II)), and nickel (Ni(II)) were examined respectively with the PCs-functionalized LSPR sensor, as shown in Fig. 5. Basically, the sensor shows a rather good linearity for the above metal ions. The

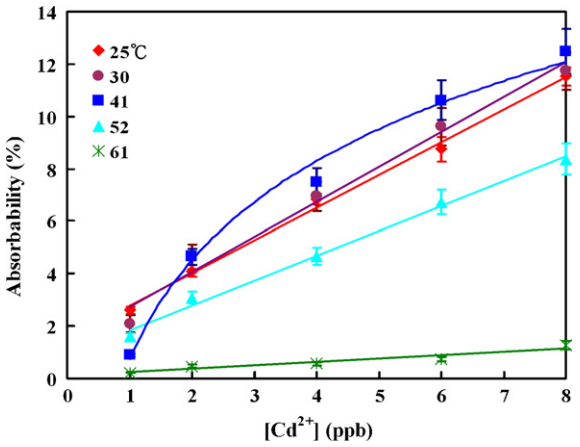

Fig. 4. Effect of detection temperature on the response for detecting 1–8 ppb Cd(II) in pH 6.5 and 0.02 M Tris buffer solution at 25 °C.

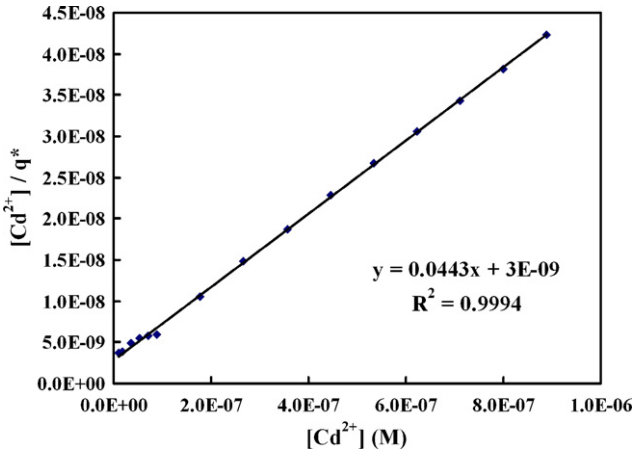

Fig. 6. The Langmuir plot of Cd(II) on the PCs-modified LSPR biosensor.

highest absorbability is less than 4% at 8 ppb Mg(II) and Cu(II), and only 2.5% for Pd(II) and Ni(II). Hence, the genetically synthesized PCs have a stronger binding ability with Cd(II).

3.4. Reactivation and stability of the sensor

The inactivated PCs after exposing to Cd(II) can be desorbed and be reused via exposing to 1 M $CaCl_2$ in 0.1 M and pH 3.6 sodium acetate buffer solution for 2 h. After nine cycles, the activity of the PCs-immobilized biosensor remains at 85%, indicating that nearly 1.6% of PCs is occupied with Cd(II) for each run. Besides, there is no significant change on the calibration curve based on each initial activity of the biosensor after reactivation.

Note that protein/peptide can cause structural folding and becomes denatured if the surface water on it is not conserved after a long-time storage. D-(+)-Trehalose dehydrate, known as mycose, is an alpha-linked sugar that has the merits of rehydration and anti-oxidation to perverse the activity of protein/peptide (Higashiyama, 2002). To prove the stability of our sensor, we performed the measurement of Cd(II) using the sensors stored in 5% D-(+)-trehalose dehydrate solution at 4 °C for up to 35 days after the initial measurement. Notably, the sensor was reactivated by 1 M $CaCl_2$ in 0.1 M and pH 3.6 sodium acetate buffer solution before being restored. The result shows that the relative activity of the sensor only decayed 9.5% after being stored for 35 days. Although there is some loss of sensor activity after each cycle of use, the sensor can be recalibrated to determine the Cd(II) concentration. No significant change in the calibration curve has been observed based on each initial activity of the biosensor after reactivation. Basically, the sensor is stable and can be reused as long as the residual activity is at least 50% of the original value.

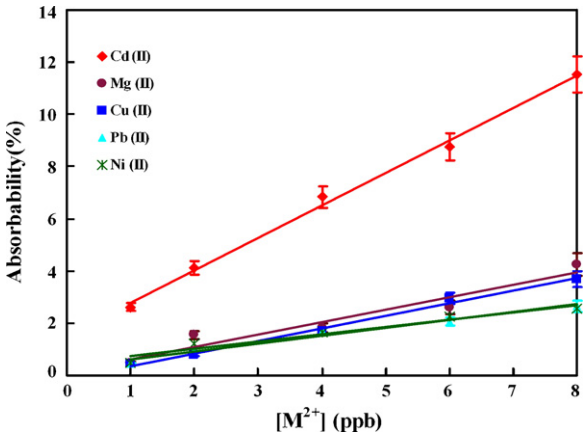

Fig. 5. Comparison of the responses between different metal ions, i.e., Mg(II), Cu(II), Pd(II) and Ni(II), through the PCs-functionalized LSPR biosensor.

3.5. Dissociation constant

In order to evaluate the binding constant of the genetically synthesized PCs chelating with Cd(II), the Langmuir isothermal adsorption equation was employed:

$$q^* = \frac{q_m c^*}{K_d + c^*} \quad (2)$$

where q^* and q_m are, respectively, the amount and the theoretical monolayer saturation capacity of adsorbed Cd(II) by the PCs-functionalized LSPR sensor at the equilibrium concentration of c^*. The dissociation constant, K_d, can be determined from a linearized form of the above equation.

$$\frac{c^*}{q^*} = \frac{K_d}{q_m} + \frac{c^*}{q_m} \quad (3)$$

Here, we can take the change in absorbability signal as the amount of adsorbed Cd(II), q^*, while the adsorption reaches the equilibrium state. Therefore, a plot of c^*/q^* versus c^* gives a straight line of slope $1/q_m$ and intercept K_d/q_m, as shown in Fig. 6. Calculation revealed a K_d value of about 6.77×10^{-8} M, which means our genetically synthesized PCs has a strong binding ability with Cd(II).

4. Conclusion

A PCs-functionalized LSPR biosensor bas been successfully developed for determining the concentration of Cd(II). The optimal incubating conditions for the immobilization of PCs on to the LSPR biosensor were found to be 71.6 μg/ml PCs in pH 7.4 PBS for 2 h. With the optimal modification, the sensor can obtain a maximal 9% change of the absorbability for detecting 1–8 ppb Cd(II), a sensitivity of 1.24 ppb^{-1}, and a LOD of 0.16 ppb. After nine cycles of deactivation with 500 ppb Cd(II) and reactivation with 1 M $CaCl_2$ in 0.1 M and pH 3.6 sodium acetate buffer solution for 2 h, the biosensor shows a good-recovery rate with 85% original activity. In addition, the biosensor produces reproducible and stable response when being used for over 35 days. The genetically synthesized PCs immobilized on the LSPR biosensor has strong binding ability to Cd(II) with $K_d = 6.77 \times 10^{-8}$ M. Conclusively, the PCs-modified LSPR

biosensor can be used for the label-free detection of Cd(II) with an excellent sensitivity.

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