Real-time molecular recognition between protein and photosensitizer of photodynamic therapy by quartz crystal microbalance sensor

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ABSTRACT

Real-time investigation of molecular recognition between protein and the photosensitizer of photodynamic therapy (PDT) was carried out by a quartz crystal microbalance (QCM) sensor integrated into a flow injection analysis (FIA) system. The photosensitizer meso-tetraakis(4-hydroxyphenyl)porphyrin (p-THPP) was immobilized on the gold electrode of the QCM chip by combining the sol-gel and self-assembly methods. Such a rapid screen analysis of molecular recognition showed that the p-THPP-immobilized sensor exhibited sensitive and specific interaction only with hemoglobin (Hb). The kinetic rate constants (kass and kdis) and the equilibrium association constant (Kd) for p-THPP–Hb interaction were calculated by linear regression. The sensing performance characteristics of the proposed sensor were investigated. The sensor showed excellent selectivity, reproducibility, and repeatability for the detection of Hb. A linear calibration plot was obtained over a range from 0.2 to 1.0 μM with a detection limit (signal/noise ratio = 3) of 0.15 μM. The response mechanism of the sensor is discussed in detail. Due to its low cost and simple manipulation, this QCM–FIA system was shown to be a highly effective method for the investigation of interaction between biomacromolecules and the PDT photosensitizer. It also provides a potential strategy for screening an efficient and less harmful photosensitizer for PDT application.

With the development of biotechnology and modern electronics, quartz crystal microbalance (QCM) sensors, due to their low cost and simple operation, have been widely used because they allow the real-time analysis of reactions without labeling requirements and provide quantitative information on the rate and equilibrium association constant [1–4]. A QCM sensor integrated with a flow injection analysis (FIA) system has the advantage of working continuously and monitoring on-line the binding of the analyte [5–8]. Owing to its shorter analysis time and less sample consumption compared with the traditional analysis methods, the QCM–FIA system could be applied to rapid analysis of specific interactions [9–12].

Photodynamic therapy (PDT) has been used as an efficient technique of cancer treatment [13–16]. This treatment involves a combination of photosensitizer drug and light, which can give rise to reactive oxygen species in the tumor environment and then lead to tumor death. Searching for high-efficient and low-toxic photosensitizers has always been one of the most important directions in the PDT field. So far as we know, it has been reported that meso-tetra(4-hydroxyphenyl)porphyrin (m-THPP) was used to destroy intrahepatic tumors with higher efficacy and fewer side effects [17]. Among the three THPP isomers (o-, m-, and p-THPP), o-THPP is toxic to skin, whereas m-THPP and p-THPP [18,19] have been shown to possess high photosensitivity and organic selectivity and been used as PDT photosensitizers for anticancer treatment. Spectrofluorometric and spectrophotometric methods are usually used for the study of the interaction between protein and PDT photosensitizers [20–22]. Compared with these two traditional methods, a QCM sensor exhibits unique advantages such as miniaturization, less sample consumption, short analysis time, and reusability. However, to our knowledge, a QCM sensor for real-time investigation on molecular recognition between protein and PDT photosensitizer has not been reported.

In this study, a QCM–FIA system was first used for real-time monitoring of molecular recognition between protein and a cancer PDT photosensitizer. The photosensitizer meso-tetraakis(4-hydroxyphenyl)porphyrin (p-THPP) was modified on the gold electrode of piezoelectric quartz crystal surface by combining sol-gel and self-assembly techniques. The interaction between the immobilized p-THPP and protein in solution could be monitored on-line as a change in the resonant frequency of the modified crystal. The
sensor only exhibited specific response to hemoglobin (Hb). The sensing performance characteristics of the proposed sensor were investigated. The response mechanism of the sensor is discussed in detail. Fluorescence and circular dichroism (CD) techniques were also employed for proving the interaction between p-THPP and Hb. This approach was of benefit to examine the side effects of the photosensitizer.

Materials and methods

Materials

2-Mercaptoethanol and titanium(IV) n-butoxide (Ti(O-2Bu)4, 99.0%) were purchased from Aldrich (St. Louis, MO, USA). p-THPP was prepared and purified [23]. Hb, trypsin, lysozyme, globulins, ribonuclease, human serum albumin (HSA), albumin egg, bovine serum albumin (BSA), pepsin, transferrin, myoglobin, and cytochrome c were purchased from Sigma (St. Louis, MO, USA). Unless otherwise noted, all solutions of proteins (concentration of stock solution: 0.1 mM) were in 10 mM phosphate-buffered solution (PBS, pH 7.4). All other chemicals were of analytical reagent grade, and deionized water was used throughout. All solutions prepared were filtered (0.45 μm) prior to use.

Methods

Immobilization of p-THPP on piezoelectric quartz crystal

All QCM measurements were performed on a Q-Sense E4 sensor (Q-Sense, Gothenburg, Sweden). All QCM chips (gold-coated quartz crystals) were first cleaned in an ultraviolet (UV)/ozone Tip-Cleaner (BioForce Nanosciences, Ames, IA, USA). After this cleaning treatment, the immobilization of p-THPP on the gold electrode of piezoelectric quartz crystal surface was performed according to the following procedure [24]. First, the gold electrode was immersed for 24 h in a 10-mM 2-mercaptopoethanol solution in ethanol, followed by rinsing with ethanol and deionized water and drying with N2 gas. Second, the mercaptobenzoate-modified gold electrode was immersed in a 100-mM Ti(O-2Bu)4 solution in 1:1 (v/v) toluene/ethanol for 5 min at 40 °C, rinsed thoroughly with ethanol to remove the physically adsorbed Ti(O-2Bu)4, immersed in deionized water for 1 min at 40 °C to generate surface hydroxyl groups, and then dried with N2 gas flushing. Third, the electrode was immersed in a 1-mM p-THPP solution in ethanol for 10 min at 40 °C, rinsed with deionized water, and dried with N2 gas. Repeating the second and third steps of the procedure, multilayer films of alternate molecular layers of titanium oxide and photosensitizer p-THPP could be formed. Fourth, the electrode was immersed in a 10-mM n-butyl mercaptan solution in ethanol to block the residual reacting sites for the prevention of nonspecific binding.

As a control, non-p-THPP-modified sensor was prepared by the first, second, and fourth steps of the procedure described above.

QCM sensor for investigation on interaction between p-THPP and protein

The fresh-coated quartz crystal was mounted in the flow-through cell and rinsed with the loading solution (10 mM PBS, pH 7.4) continuously until the frequency had stabilized under flow condition (60 μl/min). By means of an injection valve, 600-μl aliquots of different protein solutions were injected into the fluid system. The curves of permanent frequency shifts versus time were recorded, and the binding process was monitored in real time. For each concentration, when the frequency change remained unchanged, the adsorption equilibrium was attained and the corresponding frequency value was recorded. The frequency change was the difference between the initial value and the adsorption equilibrium value.

Evaluation of binding parameters by spectrofluorometric study

The interaction between p-THPP and Hb was studied spectrofluorometrically on an F-4500 spectrofluorometer (Hitachi, Tokyo, Japan) at room temperature (25 °C). The Hb fluorescence was titrated by adding p-THPP from a concentrated stock solution (0.4 mM) to 1 ml of the Hb (9.0 μM) with excitation and emission slits of 5.0 nm. The fluorescence intensity was measured at the maximal excitation wavelength of 282 nm and the maximal emission wavelength of 331 nm. Spectra were recorded after baseline correction.

Circular dichroism measurements

CD measurements of Hb were performed on a J-600 spectropolarimeter (Jasco, Tokyo, Japan) using a quartz cuvette with a pathlength of 5 mm. Molar ellipticity, [θ] values, were obtained using the following relation [25]:

\[ [\theta] = \frac{M,W}{l \cdot c} \]

fraction of α-helix = (([θ]222 + 2340)/(−30300)),

where [θ]222 is the ellipticity at 222 nm.

Results and discussion

Interaction of p-THPP-immobilized sensor with different protein

Prior to sample injection, the p-THPP-modified quartz crystal was mounted in the flow-through cell and rinsed with PBS until a steady baseline was obtained. Then 600-μl aliquots of different protein solutions (prepared in 10 mM PBS, pH 7.4) were injected into the fluid system to monitor the interactions that could occur on p-THPP-modified quartz crystal surface. The p-THPP-modified sensor showed a negligible frequency change for PBS. When different protein solutions with the same concentration were injected into the fluid system, no appreciable frequency changes were ob-

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**Fig. 1.** Interaction of photosensitizer p-THPP-modified sensor with different protein solutions with the same concentration as Hb (1.0 μM).
served except for Hb (1.0 μM). A remarkable frequency decrease for PBS in the presence of Hb was observed, clearly demonstrating that the p-THPP-modified sensor could interact with Hb. Also the concentrations of other protein solutions were twice as much as the concentration of Hb, no noticeable frequency changes were observed (see Fig. 1). In addition, the response to Hb with a non-p-THPP-modified sensor was examined. No observable frequency change was shown, suggesting that a molecular recognition between p-THPP and Hb indeed existed. Moreover, the frequency change of the sensor was gradually enhanced with increasing Hb concentrations (Fig. 2). After every binding measurement, 0.01 M HCl solution was adopted to regenerate the sensor until the frequency gradually returned back to the initial level. Such a QCM–FIA analysis provided a simple and cheap method to study the interaction between photosensitizer porphyrins and biomacromolecules.

**Sensing performance of p-THPP-modified sensor**

**Effect of sensing film thickness on frequency response of sensor**

One merit of multilayer films is that the thickness of the film can be precisely controlled by regulating the number of layers. Therefore, it is interesting to evaluate the effects of the film thickness on the Hb binding properties. We investigated the frequency change originating from the combined Hb as a function of the layer numbers after Hb solution (0.4 μM) was injected. With the increase of layer numbers from 1 to 9 (Fig. 3), the frequency change of the sensor nearly kept constant (relative standard deviation [RSD] = 1.0%, n = 7). This indicated that the loading amount of Hb complexed with p-THPP did not change with the increasing layer numbers, although the loading amount of p-THPP was enhanced in the thicker film. These phenomena confirmed that p-THPP involved in the interaction with Hb was located on the surface of the film rather than in the inner layers.

**Selectivity of p-THPP-modified sensor**

Some biomacromolecules existing in biological samples were chosen for the study on selectivity of the Hb sensor. Albumin was selected because it coexists in serum with Hb. Transferrin was a ferrous protein in blood plasma. Myoglobin and cytochrome c were chosen because they contained the same haemachrome group as Hb. A foreign species was considered not to interfere with measurement if a relative error caused by it was less than 5% in the determination of 1.0 μM Hb. The critical concentration of each interfering protein in excess of which the frequency signal was disturbed is given in Table 1. The results presented in the table reveal that the species caused no interference when the coexisting amount was 2 times for HSA or BSA, 3 times for albumin egg, 1.7 times for transferrin, 3.8 times for myoglobin, or 5.5 times for cytochrome c. The p-THPP-modified QCM sensor showed excellent selectivity.

**Reproducibility, repeatability, and reversibility of p-THPP-modified sensor**

The repeatability and reversibility of the p-THPP-modified sensor in the determination of Hb were evaluated by repetitively exposing the quartz crystal to Hb solution, 0.01 M HCl, and PBS (pH 7.4). Using a QCM chip, the RSDs in the determination of two different Hb concentrations were found to be 2.7% (0.4 μM, n = 3) and 1.3% (0.8 μM, n = 3), respectively. The Hb complexed with p-THPP could be eluted out of the sensing film completely, demonstrating the excellent repeatability and reversibility of the

![Fig. 2. Effect of Hb concentration on frequency response.](image)

![Fig. 3. Effects of layer numbers on frequency change of p-THPP-modified sensor. The concentration of Hb was fixed at 0.4 μM.](image)

![Fig. 4. Frequency responses in determination of Hb by repetitively exposing quartz crystal to 0.4 μM Hb solution, 0.01 M HCl, and PBS (pH 7.4).](image)
sensor. Fig. 4 shows the frequency response in the determination of Hb by repetitively exposing the quartz crystal to 0.4 μM Hb solution, 0.01 M HCl, and PBS (pH 7.4).

Also, three QCM chips of the same type were modified, and the response reproducibility was examined. Using the modified QCM chip of different batches, the RSDs in the determination of two different Hb concentrations were found to be 4.9% (0.4 μM, n = 3) and 4.0% (0.6 μM, n = 3), respectively. This convincingly proved the reproducibility of the sensor. Superior reproducibility, repeatability, and reversibility manifested the successful fabrication of the Hb QCM sensor.

A linear range for Hb covered from 0.2 to 1.0 μM. The detection limit was 0.15 μM (signal/noise [S/N] ratio = 3). The linear calibration equation was \( \Delta f = 22.865c - 2.085 \) with a correlation coefficient \( r = 0.9916 \) by three repetitive Hb determinations.

**Estimation of equilibrium association constant for interaction of p-THPP with Hb**

Kinetic and equilibrium association constant obtained from QCM–FIA

Because the QCM–FIA system is a continuous and real-time detector, it is possible to assess the kinetics of interaction. The reaction between the immobilized compound (B) and the molecule (A) in solution is often assumed to follow pseudo-first-order kinetics [26,27]. For the reversible interaction, \( A + B \leftrightarrow AB \), the formation rate of the product (\( AB \)) at time \( t \) may be written as

\[
\frac{d[AB]}{dt} = k_{ass} \cdot |A| \cdot |B| - k_{diss} \cdot |AB|
\]

(1)

where \( k_{ass} \) is the association rate constant and \( k_{diss} \) is the dissociation rate constant. After some reaction time \( t \), \( |B| = |B|_0 - |AB| \).

Substituting into Eq. (1) gives

\[
\frac{d[AB]}{dt} = k_{ass} \cdot |A| \cdot (|B|_0 - |AB|) - k_{diss} \cdot |AB|
\]

(2)

where \( |B|_0 \) is the concentration of B at \( t = 0 \).

Considering the Sauerbrey equation (the decrease of frequency \( \Delta f \) is directly proportional to the attached mass) and using \( \Delta f_m \) as the frequency change after a complete saturation of the crystal surface with A, the concentration of the free B is proportional to \( (\Delta f_m - \Delta f) \) and the concentration of the complex \( AB \) is proportional to \( \Delta f \). Thus, Eq. (2) can be expressed as

\[
\frac{d(\Delta f)}{dt} = k_{ass} \cdot (\Delta f_m - \Delta f) \cdot c - k_{diss} \cdot \Delta f.
\]

(3)

Thus, it can be transformed as

\[
\frac{d(\Delta f)}{dt} = -(k_{ass} \cdot c + k_{diss}) \cdot \Delta f + k_{ass} \cdot \Delta f_m \cdot c.
\]

(4)

where \( c \) is the concentration of the free A, kept constant in a continuously flowing solution.

By measuring the binding curves (\( f - t \)) determined for several concentrations \( c \), all of the desired parameters \( k_{ass} \), \( k_{diss} \), and \( \Delta f_m \) could be obtained. The equilibrium association constant \( K_A \) for the AB complex can be obtained as a ratio:

\[
K_A = \frac{k_{ass}}{k_{diss}}.
\]

Based on the approach described above, \( k_{ass} \), \( k_{diss} \), and \( K_A \) were calculated for three determinations and are given with standard deviations as follows:

\[
k_{ass} = (9.03 \pm 0.04) \times 10^3 \text{ M}^{-1} \text{ s}^{-1}
\]

\[
k_{diss} = (7.11 \pm 0.23) \times 10^3 \text{ s}^{-1}
\]

\[
K_A = (1.27 \pm 0.17) \times 10^6 \text{ M}^{-1}.
\]

**Binding parameters from spectrofluorometric study**

To validate the reliability of the QCM–FIA system, the spectrofluorometric method was used to estimate the binding parameters of the interaction between p-THPP and Hb. Binding parameters were determined from the quenching of Hb fluorescence with the continuous addition of p-THPP. Quenching of Hb fluorescence intensity in the presence of the added p-THPP was measured from the change in the respective emission intensity at 331 nm. The quenching data were then analyzed to obtain the equilibrium association constant (\( K \)) and the possible number of binding sites (\( p \)).

Fig. 5 represents a linear plot of \( F_0/\Delta F \) versus \( 1/L_i \) following this equation [28]:

\[
\frac{F_0}{\Delta F} = \frac{F_0}{F_0 - F} \text{ constant if } L_i \to \infty.
\]

where \( F_0 \) and \( F \) denote the fluorescence intensity of Hb in the absence and presence of p-THPP, respectively, \( \Delta F = F_0 - F \), \( \Delta F_{max} \) represents the maximum quenched fluorescence intensity, and \( L_i \) represents p-THPP concentration. \( F_0/\Delta F_{max} \) represents the intercept of the linear plot on the \( F_0/\Delta F \) axis, and the slope gives a measure of the equilibrium association constant (\( K \)). The equilibrium association constant (\( K \)) was calculated to be \( 3.45 \times 10^5 \text{ M}^{-1} \). It is close to the value from QCM–FIA, effectively proving that the QCM–FIA method is feasible to investigate the interaction between protein and the PDT photosensitizer.

The possible number of binding sites (\( p \)) was determined from the plot of \( 1/(1 - \theta) \) versus \( L_i/\theta \) according to the following equation [28]:

\[
\frac{1}{1 - \theta} = K \cdot \frac{L_i}{\theta} - K \cdot \theta \cdot c,
\]

where \( \theta \) is the fractional saturation of p-THPP binding sites. \( \theta \) is determined from the ratio of \( \Delta F/\Delta F_{max} \) and \( c \) is the fixed concentration of Hb. The number of binding sites was estimated to be 0.275. Fractional site behavior could be explained that only a certain fraction of Hb in the solution was capable of binding p-THPP.

**Response mechanism of p-THPP-modified sensor**

Hb is composed of four subunits: two \( \alpha \) subunits and two \( \beta \) subunits. These four subunits can self-assemble an \( \alpha_2\beta_2 \) configuration in the electrolytical solution that is close to the human physiological environment.
ical environment. Every subunit in Hb molecule is made up of a peptide chain and a hemachrome molecule. The peptide chain can twist and fold to a global shape, and this special structure is called bead protein in which the hemachrome molecule is embedded. According to Hyperchem Pro (version 7.0) software developed by Hypercube (Gainesville, FL, USA), the optimal \( \text{p-THPP–Hb} \) complexation configuration is obtained based on the minimized energy. Fig. 6 gives the schematic configuration of complexation between one Hb subunit and \( \text{p-THPP} \). Hemachrome is a small molecule possessing a porphyrin structure (see hemachrome ball–stick model (a) in Fig. 6, where blue, purple, gray, white, and red balls represent nitrogen, iron, carbon, hydrogen, and oxygen atoms, respectively). In the porphyrin group center of the hemachrome molecule, the nitrogen atoms of the four pyrrole rings coordinate with a ferrous ion. At the same time, the nitrogen of indole side chain from the 8-position histidine group (see histidine ball–stick model (b) in Fig. 6) of bead protein can coordinate with the ferrous ion above the porphyrin plane of the hemachrome molecule. The subunit structure of Hb is in a loose state under an oxygenous environment, so that the oxygen atom can easily coordinate with the ferrous ion under the porphyrin plane of the hemachrome molecule. Besides oxygen, Hb can combine with other compounds such as carbon monoxide (CO), carbon dioxide (CO\(_2\)), and cyanic ion. The association mode of these compounds is the same as that of the oxygen molecule. The only discrimination is the difference of association intensity.

When Hb solutions were injected into the fluid system, the sensor exhibited an obvious response to Hb. This fact may be interpreted as due to the formation of a complexation between the hydroxy of \( \text{p-THPP} \) (see \( \text{p-THPP ball–stick model (c) in Fig. 6} \)) and the ferrous ion of hemachrome in the Hb molecule. As a contrast, the solution of Hb saturated with oxygen was injected into the fluid system, and no detectable frequency response was observed. The experiment proved that when the photosensitizer \( \text{p-THPP} \) was injected into the blood, the oxygen combined with Hb in the blood would not be displaced by \( \text{p-THPP} \). That is, the oxygen exhibited stronger interaction with Hb than did the photosensitizer \( \text{p-THPP} \). Therefore, \( \text{p-THPP} \) could not affect the biological function of Hb when it is applied in the PDT field. This further confirmed that the explanation about the interaction between \( \text{p-THPP} \) and Hb is reasonable. Also, it gives us a clue to explain the response of sensor to myoglobin and cytochrome \( c \). Although these two proteins contain the same hemachrome group as Hb, the sensor showed no observable frequency response for myoglobin and cytochrome \( c \). It was possible that the coordination number of ferrous ion group in myoglobin or cytochrome \( c \) was full, so that no residual binding sites can be used. In comparison with the oxygen molecule, the \( \text{p-THPP} \) molecule is greater. So, when the \( \text{p-THPP} \) molecule interacted with Hb, it might induce the alteration of secondary structure of Hb. At high acidity, \( \text{H}^+ \) exhibits higher affinity to Hb than does oxygen. The affinity between the Hb and the oxygen atom of \( \text{p-THPP} \) was greatly decreased with the decrease of pH; hence, Hb complexed with \( \text{p-THPP} \) could be completely eluted from the sensor.

To examine the conformational change of Hb, if any, on binding with \( \text{p-THPP} \), CD spectra were studied. Fig. 7 shows CD spectra of Hb in the absence and presence of \( \text{p-THPP} \). In the region of the wavelength from 200 to 250 nm, CD measurement of Hb gives information in relation to the conformation of secondary structure. The untreated Hb shows the spectral pattern with approximately 52% \( \alpha \)-helix content. The negative ellipticity of Hb at 222 nm was.

Fig. 6. Schematic configurations of complexation between one Hb subunit and \( \text{p-THPP} \). a. Hemachrome group ball–stick model in one Hb subunit, where blue, purple, gray, white, and red balls represent nitrogen, iron, carbon, hydrogen, and oxygen atoms, respectively; b. histidine group ball–stick model in one Hb subunit; c. photosensitizer \( \text{p-THPP} \) ball–stick model. (For interpretation of the references to color in the text description of this figure legend, the reader is referred to the Web version of this article.)
gradually reduced with the addition of p-THPP, indicating a reduction in the α-helicity of p-THPP-treated Hb. As shown in Fig. 7, when the concentration of p-THPP was 3.6 μM, meaning that the ratio of photosensitizer to protein was nearly 4, the α-helical content of Hb was reduced by approximately 38%. This result suggested that the binding of p-THPP to Hb induced the conformational change of the Hb part, further confirming the rationality of the proposed sensing mechanism. Also, this explanation is in conformity with a report that hematoporphyrin induced alteration of secondary structure of tetrameric Hb [29].

Conclusion

The QCM sensor integrated with an FIA system is a convenient and valuable tool for real-time monitoring of molecular recognition between protein and the PDT photosensitizer. Rapid screen analysis of binding between the immobilized photosensitizer and different proteins in solution clearly indicated that the photosensitizer p-THPP showed specific response to Hb. Kinetic and equilibrium association constants were evaluated. The obtained quantitative information on the binding kinetics was of benefit to understanding the interaction mechanism between Hb and the PDT photosensitizer. This kind of QCM sensor integrated with an FIA system provided a new approach for the investigation of the interaction between biomacromolecules and the PDT photosensitizer in the future. Meanwhile, it has potential for screening an efficient and less harmful photosensitizer for PDT application.

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