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Electrochemical piezoelectric quartz crystal impedance study on the interaction between concanavalin A and glycogen at Au electrodes

Liang Tan [a,b], Qingji Xie [b,*], Shouzhuo Yao [a,b,*]

[a] Key Laboratory of Chemical Biology and Traditional Chinese Medicine Research (Ministry of Education of China), College of Chemistry and Chemical Engineering, Hunan Normal University, Changsha 410081, PR China
[b] State Key Laboratory of Chemo/Biological Sensing and Chemometrics, Hunan University, Changsha 410082, PR China

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Abstract

The carbohydrate research has emerged as a "new frontier" in chemical/biological field. The binding of lectin with carbohydrate is one of the important courses of life activities. The report studies concanavalin A (Con A)–glycogen interaction on gold electrode surfaces by electrochemical piezoelectric quartz crystal impedance (EPQCI) method. The piezoelectric quartz crystal (PQC) parameters, resonant frequency shift (Δf_0) and the motional resistance change (ΔR_1), and the electrochemical impedance (EI) parameters, electrolyte resistance change (ΔR_s) and the double layer capacitance change (ΔC_s), were measured and discussed simultaneously. Two methods were adopted for measuring the Con A–glycogen association. Based on EPQCI measurement during Con A reaction with glycogen adsorbed on Au electrode, association constant K_a and the amount of the binding sites s calculated are 1.48×10^6 M^{-1} and 4.09, respectively. Based on single PQC measurement of glycogen reaction with Con A assembled on Au electrode, K_a was estimated to be 1.26×10^6 M^{-1}.
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Keywords: Concanavalin A; Glycogen; Electrochemical piezoelectric quartz crystal impedance; The association constant; Au electrode

1. Introduction

Since its successful liquid-purpose application in the very beginning of 1980s [1], the piezoelectric quartz crystal (PQC) sensor has been widely used by analysts and electrochemists due to its satisfactory performance, e.g., high sensitivity, rapid and facile operation, etc. It is well known that the Sauerbrey equation [2] displays a good linear relationship between the mass change (Δm in g) and the PQC frequency change (Δf_0 in Hz),

$$\Delta f_0 = -\frac{2f_{0_g}^2}{(\rho_q\mu_q)^{1/2}}\frac{\Delta m}{A} \quad (1)$$

where f_{0_g} is the fundamental frequency, A is a piezoelectrically active surface area, and ρ_q and μ_q are the density and shear modulus of quartz, respectively. It is worth pointing out that this equation is valid for loading and removal of thin, rigid and uniform film on the electrode surface.

In the liquid phase the PQC response is sensitive not only to mass loading but also to changes in solution density and viscosity near the electrode. Martin et al. [3] reported a series of equivalent circuit parameters and a modified Butterworth–Van Dyke (BVD) equivalent electrical circuit, as shown in Fig. 1, for the characterization of a quartz crystal microbalance (QCM) with simultaneous mass and liquid loading. The PQC impedance analysis is based on the modified equivalent circuit composed of a motional arm and a static arm in parallel. The motional arm contains three equivalent circuit elements in series, namely, the motional resistance R_1 ($R_1=R_q+R_L$), the motional inductance L_1 ($L_1=L_q+L_L+L_m$), and the motional capacitance C_1($C_1=C_q$). For a PQC with one face in contact with the liquid, the solution viscosity–density effects on the f_0 and R_1 can be deduced from Martin's model:

$$\Delta f_{0_L} = -\frac{f_{0_g}^{3/2}}{(\pi\rho_q\mu_q)^{1/2}}\left[(\rho_{L_2}\eta_{L_2})^{1/2}-(\rho_{L_1}\eta_{L_1})^{1/2}\right] \quad (2)$$

* Corresponding authors. Key Laboratory of Chemical Biology and Traditional Chinese Medicine Research (Ministry of Education of China), College of Chemistry and Chemical Engineering, Hunan Normal University, Changsha 410081, PR China. Tel./fax: +86 731 8865515.
E-mail addresses: tlg0686@163.com (L. Tan), xieqj@hunnu.edu.cn (Q. Xie), szyao@hunnu.edu.cn (S. Yao).

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$$\Delta R_{1_L} = -\frac{4f_{0_g}L_q(\pi f)^{1/2}}{(\bar{c}_{66}\rho_q)^{1/2}}[(\rho_{L_2}\eta_{L_2})^{1/2}-(\rho_{L_1}\eta_{L_1})^{1/2}] \quad (3)$$

where f_0 is the resonance frequency and $f_0=1/[2\pi(L_1C_1)^{1/2}]$, Δf_{0_L} and ΔR_{1_L} are the changes in the resonant frequency and motional resistance of PQC due to variations in the solution density (ρ_L) and viscosity (η_L), f_{0_g} is the resonant frequencies of the PQC in air, ρ_q is the density of quartz (2648 kg m^{-3}), μ_q is the shear modulus for AT-cut quartz (2.947×10^{10} N/m^2), $\bar{c}_{66}$ is the piezoelectrically stiffened elastic constant (2.957×10^{10} N/m^2) [4], and the subscripts of L_1 and L_2 denote ρ_L and η_L of states (1) and (2), respectively.

Combination of Eqs. (2) and (3) yields:

$$\Delta R_{1_L} = -\frac{4\pi L_q\sqrt{f\mu_q}}{\sqrt{\bar{c}_{66}f_{0_g}}}\Delta f_{0_L} \quad (4)$$

The relationship between Δf_0 and ΔR_1 due to net changes in solution density and viscosity can thus be obtained. For the 9 MHz crystal used in this work, the slope of Δf_0 versus ΔR_1 calculated from Eq. (4) is about -10 Hz Ω^{-1}. The PQC-based biosensor has been widely used for analyses of protein [5], nucleic acid [6], and enzyme [7], and for immunoassay [8], etc. In addition, the PQC sensor has been combined with many other analytical techniques, e.g., electrochemical methods, spectrophotometry, for acquiring multi-dimensional and *in situ* information in chemical and/or biological events at/near the PQC electrode [9–14].

Electrochemical impedance spectroscopy (EIS) is a powerful tool for investigating the interfacial properties of surface-modified electrode [15]. The typical electrochemical interface can be represented as an electrical circuit composed of the electrolyte resistance (R_s) in series with the parallel combination of the double layer capacitance (C_s) with the Faradaic impedance (Z_f). The electrochemical complex impedance (Z) is represented as a sum of the real (Z_{re}) and imaginary (Z_{im}) parts ($Z=Z_{re}-jZ_{im}$), since electrochemical interface can be simplified as a series RC circuit for a given higher frequency. R_s and C_s can be calculated out from Z_{re} and Z_{im} according to $R_s=Z_{re}$ and $C_s=1/(2f\pi Z_{im})$. The information of surface-modification on an electrode can be effectively obtained by measurement of electrochemical impedance (EI) at a proper frequency.

Beside nucleic acids and proteins, carbohydrates play critical roles in biological systems and their study and characterization have become increasingly important. Glycoconjugates serve as important parts in cellular recognition, transmission of information, immunity, and tumorigenesis [16]. Glycogen as one of glycoconjugates is extensively distributed in animal tissues including liver, skeletal muscle, heart, and brain. Glycogen functions as an energy-storage form of glucose in animal cells, which resembles the mission of starch in plant [17]. The content of glycogen in tissues is regarded as a kind of signal reflecting the state of health, whose determination is thus of obvious importance for the early diagnosis of related diseases [18]. Some methods, such as iodine binding assay [19], enzymatic method [20], and light scattering technique [21], have been applied to detect glycogen. Concanavalin A (Con A), derived from jack bean, is one of the best characterized lectins. It exhibits high affinity for α-D-glucose, α-D-mannose, and glycogen [22]. The existent structure of Con A is dependent on the pH conditions of solutions. At lower pH (below 5.9), Con A is a dimer. At higher pH (above 6.9), it is a tetramer. So Con A has four identical binding sites for sugar per molecule at physiological pH solution. The Con A–sugar combination, being very similar to the well-known antigen–antibody combination, has also been used for constructing sugar-containing thin films for biological and chemical studies [23–26].

The present report gives a detailed account of piezoelectric quartz crystal impedance (PQCI) analysis in combination with cyclic voltammetry (CV) and electrochemical impedance (EI) for studying Con A–glycogen interaction on gold electrode surface. Both the association constant K_a and the amount of the binding sites s are obtained by the electrochemical piezoelectric quartz crystal impedance (EPQCI) system.

2. Experimental

2.1. Materials

Concanavalin A (biochemical grade), glycogen (biochemical grade, FW 100 000) and bovine serum albumin (BSA) were purchased from Shanghai Yuanju Biological Technique Co., Ltd and used as received. The solutions of Con A and glycogen were prepared by dissolving them in doubly-distilled water and stored in the refrigerator at 4 °C before use. A pH 7.2 phosphate buffer solution (PBS) was prepared by mixing 72 ml of 0.2 mol L^{-1} Na_2HPO_4 with 28 ml of 0.2 mol L^{-1} KH_2PO_4. All other chemicals were of analytical grade, and all aqueous solutions were prepared in distilled water, which was produced with a double-distillation quartz set.

2.2. Apparatus

The setup for the combined PQCI and EI measurements included an HP4395A Network/Spectrum/Impedance analyzer, a CHI 660 electrochemical analyzer and two IBM-compatible

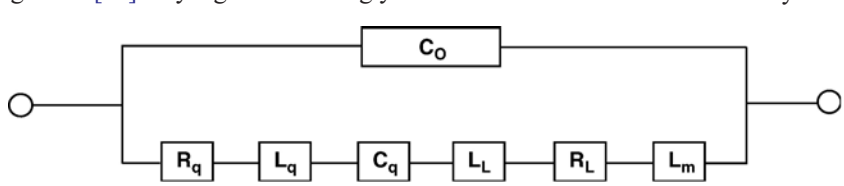

Fig. 1. A modified Butterworth–Van Dyke equivalent electric circuit for quartz crystal resonance, where R_q, L_q and C_q are motional resistance, motional inductance and motional capacitance of unperturbed crystal, respectively. R_L and L_L are the motional resistance and motional inductance due to liquid loading, respectively. L_m is the motional inductance due to mass loading. C_0 is the static capacitance.

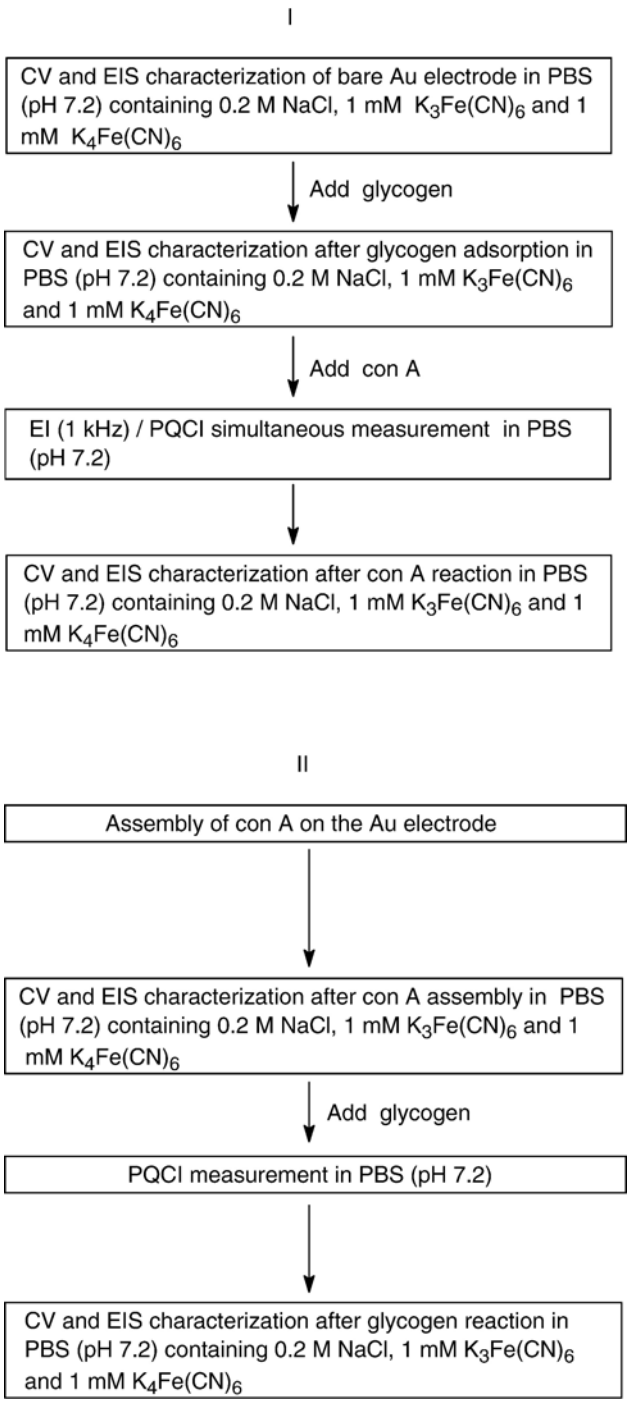

Fig. 2. Schematic representation of two experimental protocols taken in this work.

personal computers (PC). AT-cut 9 MHz piezoelectric quartz crystals (12.5 mm in diameter) with gold electrodes (6 mm in diameter) on both sides were used, with only one side in contact with the test solution. Based on the simultaneous fitting of synchronously recorded conductance (G) and susceptance (B) for the PQC resonance to the Butterworth–Van Dyke (BVD) equivalent circuit, the equivalent circuit parameters and PQC resonant frequencies were acquired real time by a user program written in Visual Basic 5.0, which also controlled the HP 4395A analyzer. The gold electrode on one PQC side was used as the working electrode (WE). A saturated KCl calomel electrode (SCE) with a supporting electrolyte salt bridge served as the reference electrode (RE) and all potentials in this work were cited with respect to it. A large-area platinum plate served as the counter electrode (CE). A conventional glass three-electrode electrolytic cell of a volume of ca. 50 ml was used. The test solution (30 ml) was stirred at a constant rate with a magnetic stirring bar. All experiments were performed at room temperature.

2.3. Measurement procedures

To remove possible contamination, the gold electrode surface was cleaned with Piranha solution (30% H_2O_2: H_2SO_4 1:3). After being rinsed thoroughly with doubly-distilled water, gold electrode was subjected to a voltammetric treatment in 0.2 M $HClO_4$ aqueous solution until cyclic voltammograms were reproducible. Then the surface was thoroughly rinsed and blown dry with a stream of nitrogen gas prior to use.

The experimental protocols are illustrated in Fig. 2. Two kinds of methods were adopted. One is the measurement of parameters during the Con A reaction with glycogen adsorbed on bare Au electrode (I). The other is the investigation for the glycogen reaction with Con A assembled on Au electrode (II). The ferricyanide/ferrocyanide couple was employed as electrochemical probes for CV and EIS studies. The experimental procedures for estimation of the association constant and the amount of the binding sites will be given in captions of related figures and text. All experiments were conducted at room temperature.

2.4. Assembly of Con A onto gold electrode

As shown in Fig. 3, the freshly cleaned PQC electrode was immersed into 0.01 mol L^{-1} cysteine (Cys) aqueous solution overnight. The Cys-modified PQC electrode was then immersed in 2.5% glutaraldehyde (GA) aqueous solution for 2 h. Subsequently, the crystal was washed with distilled water and then nitrogen-dried, followed by dropping 10 μl of 5 mg ml^{-1} Con A aqueous solution on the dry Cys/GA-modified gold surface. By the time the solvent was evaporated, the crystal was washed with PBS (pH 7.2) and then nitrogen-dried.

3. Results and discussion

3.1. Adsorption of glycogen onto bare Au electrode and subsequent reaction of Con A

Fig. 4(A) shows time courses of simultaneous responses of the resonant frequency (Δf_0) and the motional resistance (ΔR_1) during immobilization of glycogen, blocking with BSA and then binding of Con A to immobilized glycogen on bare Au electrode in PBS buffers (pH 7.2). It is found that Δf_0 decreased slowly and kept declining all the time with addition of glycogen. Meanwhile, ΔR_1 in the bare gold case kept slow increasing. This phenomenon is similar to the behavior of starch adsorbed on QCM [27]. It implies that the glycogen adsorption on the

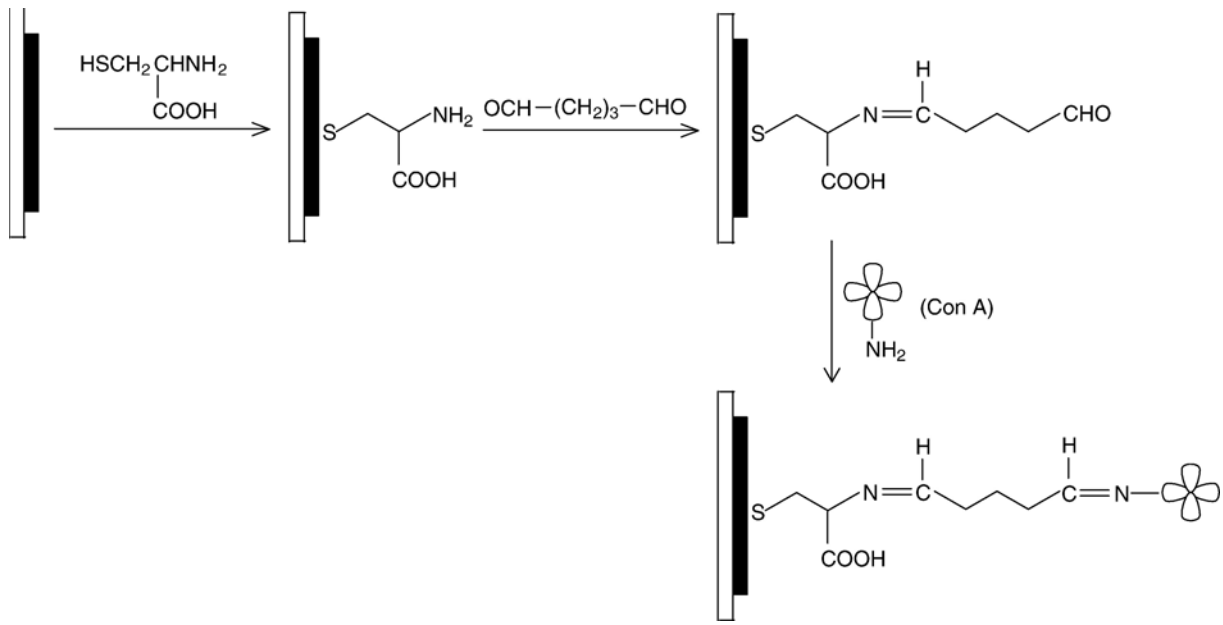

Fig. 3. Schematic representation of the Con A assembling process on the Au-QCM.

bare gold is kinetically sluggish. As a blocking reagent, BSA can inhabit the active sites for non-specific adsorption on Au electrode. Δf_0 and ΔR_1 changed immediately and became steady in short time after BSA was added, which indicates that the whole surface of Au electrode was covered by biological molecules. Subsequently, we drew a comparison between bindings of Con A to immobilized glycogen and direct adsorption of Con A on bare Au electrode in PBS buffers (pH 7.2), respectively. It is obvious that the two modes of Con A adsorption are different. Direct adsorption of Con A on bare Au electrode is a gradual course that resembles that of glycogen. And yet binding of Con A to glycogen-modified Au electrodes brings the distinct decrease of Δf_0 and ΔR_1. The R_1 response should reflect the viscous changes in the surrounding environment of the PQC [4]. For the density–viscosity effect of a 9 MHz crystal in a Newtonian liquid, the characteristic $\Delta f_0/\Delta R_1$ value should be about −10 Hz Ω^{-1} [3,28]. In other words, a ΔR_1 of 1 Ω is able to cause a Δf_0 of only about 10 Hz. The relationships between Δf_0 and ΔR_1 here are shown in Fig. 4(B). The $\Delta f_0/\Delta R_1$ (slope) values for curves 1–4 are calculated to be −41.6, −42.0, 44.2, and −43.2 Hz Ω^{-1}, respectively. It is thus concluded that the frequency shifts caused by adsorption of glycogen, BSA and Con A on Au electrode might mainly be caused by the mass effect, and Sauerbrey's rigid approximation is reasonably valid. The decrease of ΔR_1 resulted from binding of Con A to glycogen can be explained that association between them gave birth to precipitates which owns rigid structure.

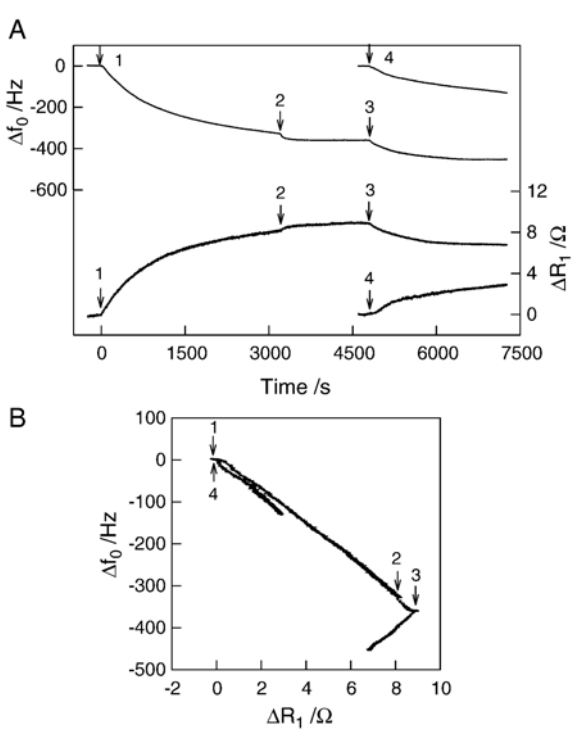

Fig. 4. (A) Time courses of simultaneous of responses of Δf_0 and ΔR_1 during immobilization of glycogen (1), blocking with BSA (2), binding of Con A to immobilized glycogen (3) and direct adsorption of Con A (4) on bare Au electrode in PBS buffers (pH 7.2). The final concentration of glycogen, BSA, and Con A were 2.63, 2.98 and 0.7396 μmol L^{-1}, respectively. (B) shows relationships of Δf_0 versus ΔR_1 for curves (1), (2), (3) and (4).

Fig. 5 exhibits the cyclic voltammograms and electrochemical impedance spectroscopy for bare Au electrode before and after glycogen and Con A were added. After glycogen adsorption, peak currents were a little decreased, while the peak-to-peak separation and the Nyquist diameter increased a little. Glycogen molecules are highly hydrophilic and have branched and loose structure, and they may be randomly adsorbed on the gold electrode surface. The ferri-/ferrocyanide probe might access to the bare electrode moieties with a certain freedom, and thus the glycogen adsorbed imposed little influence on the electrode activity. Binding of Con A to glycogen adsorbed on

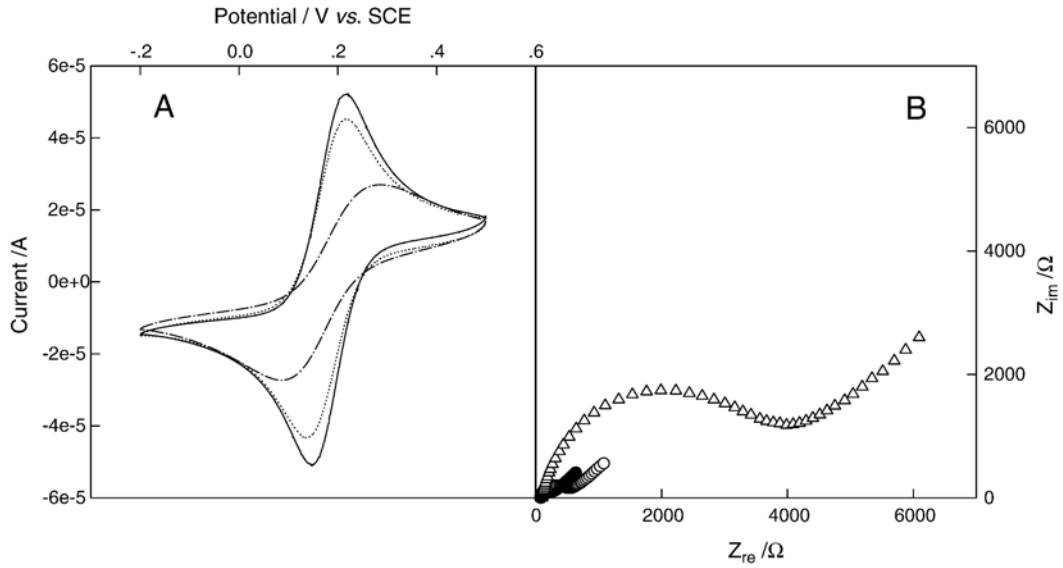

Fig. 5. Cyclic voltammograms (A) for bare (solid line), glycogen- (dotted line) and glycogen/Con A-modified (dash dotted line) Au electrodes as well as electrochemical impedance spectra (B) for bare (●), glycogen- (○) and glycogen/Con A-modified (△) Au electrodes in a PBS solution (pH 7.2) containing 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, and 0.2 M NaCl. (A) dE/dt=20 mV s^{-1}; (B) 100 kHz~5 mHz, 10 mV rms, 0.18 V versus SCE.

the electrode surface and formation of precipitates decreased the electrochemical activity to a certain extent, as revealed by some lowered peak currents, and certain increased peak-to-peak separation and Nyquist diameter for the curve in Fig. 5.

Because the glycogen–Con A interaction resembles an antibody–antigen reaction, estimation of the association constant K_a between glycogen and Con A and the amount of the binding sites s of Con A can be achieved according to the following equations [29]:

$$\frac{c_F}{\Delta C_s} = \frac{1}{K_a \Delta C_s^0} + \frac{c_F}{\Delta C_s^0} \tag{5}$$

$$\frac{c_F}{\Delta f_0} = \frac{1}{K_a \Delta f_0^0 s} + \frac{c_F}{\Delta f_0^0} \tag{6}$$

where K_a is the association constant; s is the amount of the binding sites of the antigen (or Con A) molecule which recognize specific areas of the antibody (or glycogen), namely, the valence of antigen (or Con A); ΔC_s and Δf_0 are the capacitance and the frequency changes measured at each antigen (or Con A) concentration, respectively; ΔC_s^0 and Δf_0^0 are the capacitance and the frequency changes measured at saturation, respectively; and c_F is the concentration of free antigen (or Con A) in solution. Since the concentration of associated antigen (or Con A) is far smaller than that of the initial antigen (or Con A) in solution c_P, c_F is approximately equal to c_P.

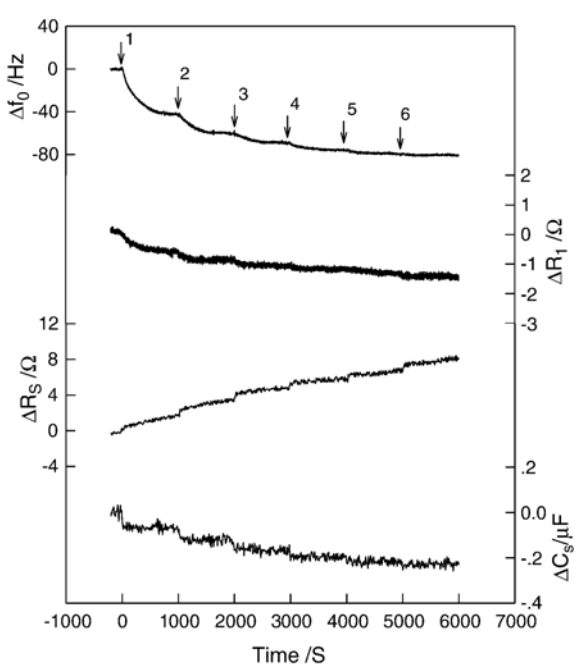

Fig. 6. Time courses of simultaneous of responses of Δf_0, ΔR_1, ΔR_s, and ΔC_s during the Con A reaction with glycogen adsorbed on bare Au electrode in PBS buffers (pH 7.2). Arrows indicate successive additions of 2 mg mL^{-1} Con A solution (200 μL for each addition) to a final concentration of 0.1274 (1), 0.2531 (2), 0.3771 (3), 0.4995 (4), 0.6204 (5), 0.7396 μmol L^{-1} (6), respectively. Electrochemical impedance measurements: 1 kHz, 10 mV rms, and at open circuit potential.

Fig. 6 shows the real-time and simultaneous monitoring of the resonant frequency shift (Δf_0), the motional resistance change (ΔR_1), the electrolyte resistance change (ΔR_s), and the double layer capacitance change (ΔC_s) due to the successive Con A reaction with glycogen adsorbed on bare Au electrode in PBS buffers (pH 7.2). Δf_0 and ΔR_1 exhibited ladder-like decrease owing to the PQC mass effect caused by glycogen–Con A precipitates. On the other hand, changes of ΔR_s and ΔC_s could demonstrate alteration of electrical properties, such as conductance and dielectric constant, in the solution and near the electrode surface. Parameters of K_a, s, ΔC_s^0 and Δf_0^0 of best fit were obtained by the linear regression program embedded in the SigmaPlot Graphing Software V2.0 based on Eqs. (5) and (6).

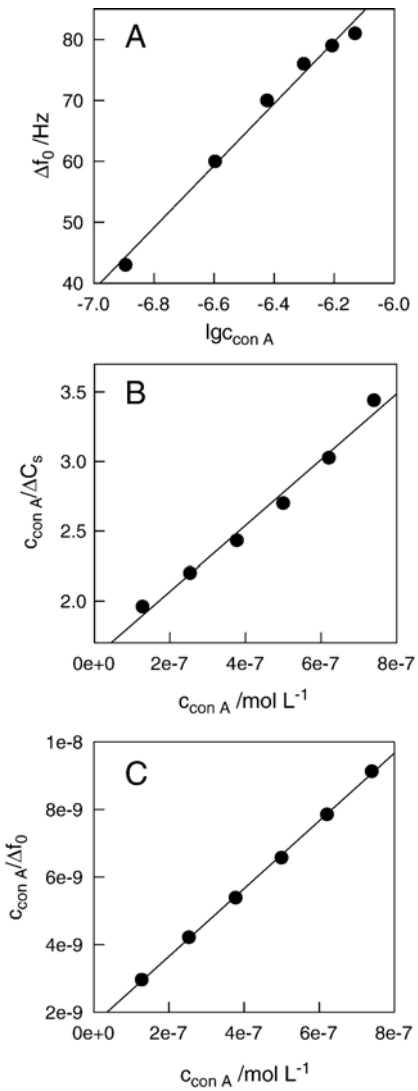

Fig. 7. (A) Calibration curve obtained for the glycogen-modified PQC sensor to Con A solutions at different concentrations. (B) and (C) represent linear transformation of relative data in Fig. 6, see text for further explanation.

Fig. 7(A) shows calibration curve of Δf_0 versus lg$c$ at different Con A concentrations. The regression equation is $\Delta f_0 = 50.9\lg c + 395$, with linearity correlation coefficient of 0.9886. Fig. 7(B) and (C) represents transformation of relative variables presented in Eqs. (5) and (6), i.e. $c_F/\Delta C_s$ versus c_F and $c_F/\Delta f_0$ versus c_F, which possess good linear relationships. The regression equations are $y_1 = 2.36\times10^6 x_1 + 1.60$ and $y_2 = 0.01x_2 + 1.65\times10^{-9}$, with linearity correlation coefficients of 0.9844 and 0.9994, respectively. According to the slope and intercept, K_a and s calculated are 1.48×10^6 M^{-1} and 4.09, respectively.

Referring to the analysis of binding ratio of antibody and antigen performed by Su, et al. [30], the frequency changes caused by glycogen immobilization ($\Delta f_{0,\mathrm{glycogen}}$) and those caused by the saturated reaction of Con A with glycogen-coated surface ($\Delta f_{0,\mathrm{Con\ A}}$) can be used to estimate the interaction ratio of glycogen and Con A by using the Eq. (1). Because the molecule weights of glycogen and Con A are 100,000 and 104,000 (tetramer), respectively, the ratio of total amount of glycogen immobilized onto the crystal (N_{glycogen}, mol) to the reaction amount of Con A bound ($N_{\mathrm{Con\ A}}$, mol) to the coated glycogen can be obtained using Eq. (7):

$$\frac{N_{\mathrm{glycogen}}}{N_{\mathrm{conA}}} = \frac{\dfrac{\Delta f_{0,\mathrm{glycogen}}}{FW_{\mathrm{glycogen}}}}{\dfrac{\Delta f_{0,\mathrm{conA}}}{FW_{\mathrm{conA}}}} \tag{7}$$

The result indicates that the ratio of N_{glycogen} to $N_{\mathrm{Con\ A}}$ is (327/100,000)/(81/104,000) ≈ 4.20, which indicates that one molecule of Con A can approximately bind with four molecules of glycogen. It is basically coincident with the result calculated by Eqs. (5) and (6) (s = 4.09).

3.2. Glycogen reaction with Con A assembled on Au electrode

Each Con A-monomer consists of 237 amino acids [31], so Con A can easily react with GA. The procedures for assembly of Con A on the PQC gold electrode by Cys and GA are depicted in Fig. 2.

It is found that the signals of the double layer capacitance change (ΔC_s) due to the successive glycogen reaction with Con A assembled on bare Au electrode in PBS buffers (pH 7.2) was quite faint. It is inferred that assembly of Con A probably has an impact on EI measurement. As a result, single PQC measurement was applied. Fig. 8 exhibits the frequency shifts of Con A-assembled Au electrode after successive additions of glycogen. The absolute values of Δf_0 are proportional to the common logarithm of glycogen concentration from 0.332 to 1.96 μmol L^{-1}. Satisfactory linear relationships, $\Delta f_0 = 96.7\lg c + 678$, with high regression

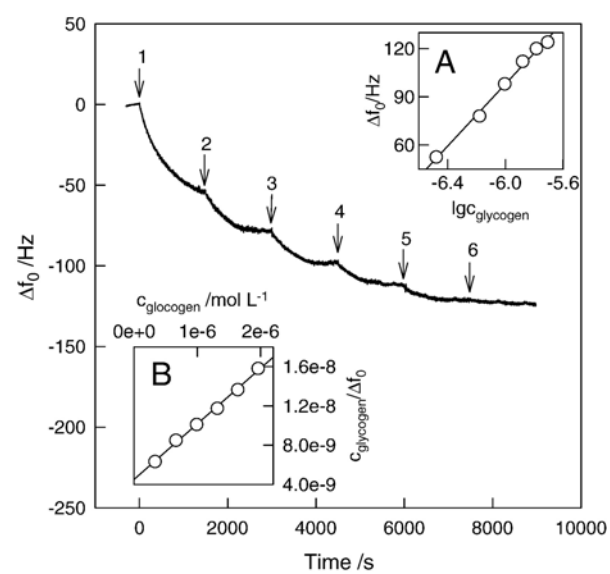

Fig. 8. Time course of response of Δf_0 during the glycogen reaction with Con A assembled on Au electrode in PBS buffers (pH 7.2). Arrows indicate successive additions of 10 mg mL^{-1} glycogen solution (100 μL for each addition) to a final concentration of 0.3322 (1), 0.6623 (2), 0.9901 (3), 1.3158 (4), 1.6393 (5), 1.9608 μmol·L^{-1} (6). Insert (A) indicates calibration curve obtained for the Con A-assembled PQC sensor to glycogen solutions at different concentrations. Inset (B) indicates linear relationships of $c_{\mathrm{glycogen}}/\Delta f_0$ versus c_{glycogen}.

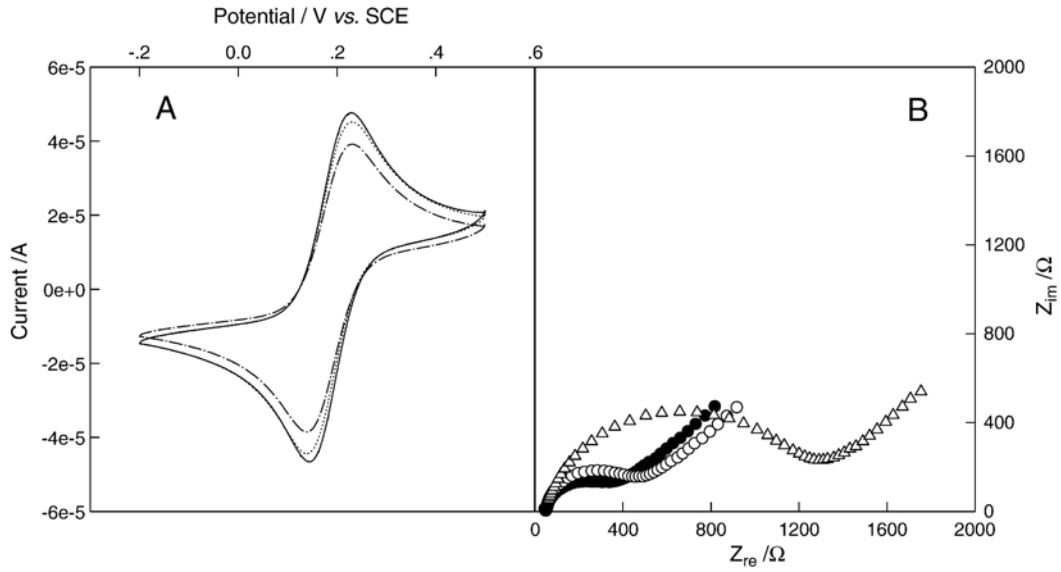

Fig. 9. Cyclic voltammograms (A) for bare (solid line), Con A-assembled (dotted line) and Con A/glycogen-modified (dash dotted line) Au electrodes as well as electrochemical impedance spectra (B) for bare (●), Con A-assembled (○) and Con A/glycogen-modified (△) Au electrodes in a PBS solution (pH 7.2) containing 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, and 0.2 M NaCl. (A) dE/dt=20 mV s^{-1}; (B) 100 kHz~5 mHz, 10 mV rms, 0.18 V versus SCE.

coefficient, 0.9945, were obtained. Optimization of the experimental conditions may find analytical applications. Inset (B) of Fig. 8 indicates linear relationships between $c_{glycogen}/\Delta f_0$ and $c_{glycogen}$ displayed in Eq. (6). K_a, 1.26×10^6 M^{-1}, is obtained since $K_a\times s$ calculated is 1.26×10^6 M^{-1} as well as the valence of glycogen (=1) is considered. The value of K_a by the method of glycogen reaction with Con A assembled on Au electrode is basically equal to that by the method of Con A reaction with glycogen adsorbed on bare Au electrode. The dissociation constant K_D ($K_D=1/K_a$) could be estimated to be 0.8 μM, which is little bigger than K_D of yeast mannan–Con A compound (0.4 μM) [32]. It seems the association of Con A with glycogen is less compact than the mannan–Con A binding. It may be the steric hindrance caused by glycogen macromolecules that has some influence on the combination between Con A and glycogen.

As shown in Fig. 9, Con A-assembled gold electrode was of electrochemical characteristics similar to the glycogen-adsorbed gold electrode (Fig. 5). It suggests that the spacing between the Con A molecules and gold substrate due to the presence of short and hydrophilic Cys/GA chains allow the facile penetration of small ferri-/ferrocyanide molecules to access to the bare gold moieties. Activity and conductivity of the electrode were further impaired after glycogen associate with Con A assembled on Au electrode. Unlike the case given in Fig. 5, here the effects of adsorption of glycogen and the glycogen–Con A adduct on activity and conductivity of the electrode is less because the more incompact space between the precipitates and gold substrate accommodates more ferri-/ferrocyanide molecules.

4. Conclusion

The work reveals that the association between glycogen and Con A can yield rigid-structure precipitates that affect activity and conductivity of Au electrode to a certain extent. Simultaneously obtained parameters by EPQCI measurement during Con A reaction with glycogen adsorbed on Au electrode were used to estimate the association constant of the glycogen–Con A interaction K_a, 1.48×10^6 M^{-1} and the amount of the binding sites s, 4.09, respectively. PQC measurement of glycogen reaction with Con A assembled on Au electrode deduced K_a is 1.26×10^6 M^{-1}. Comparability of K_a values by two methods shows validity of EPQCI measurement, which indicates that this technique probably can be applied as an effective method to studies of interfacial biomacromolecular interaction, including many other lectins. In addition, quantitative analyses for relevant biomacromolecules may be achieved by the EPQCI method provided that optimal conditions for determination are investigated.

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