A simultaneous electrochemical impedance and quartz crystal microbalance study on antihuman immunoglobulin G adsorption and human immunoglobulin G reaction

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Abstract

The quartz crystal microbalance (QCM), in combination with electrochemical impedance spectroscopy (EIS), has been utilized to monitor in situ antihuman IgG (hIgG) adsorption on bare poly(o-phenylenediamine) (PPD)- and 1-dodecanethiol (C12SH)-modified Au electrodes and succeeding human IgG reaction, respectively. The resonant frequency ($f$) and the motional resistance ($R_1$) of the piezoelectric quartz crystal (PQC) as well as electrochemical impedance (EI) parameters were measured and discussed. The standard heterogeneous rate constants of the ferricyanide/ferrocyanide couple before and after the antibody adsorption and antibody–antigen reactions were determined. The results show that the amount for antibody adsorption was the greatest on the most hydrophobic (1-dodecanethiol-modified) surface, while the antibody bioactivity was almost identical on the three kinds of surfaces. Two parameters simultaneously obtained, $\Delta f$ and $\Delta C_s$ (interfacial capacitance), have been used for the first time to estimate both the association constant of the immunoreaction and the valence of antigen with satisfactory results. The proposed method may find wide application in interfacial biochemistry studies for its

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advantages in providing real-time multidimensional piezoelectric and electrochemical impedance information.
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**Keywords:** Quartz crystal microbalance; Electrochemical impedance spectroscopy; The association constant of immunoreaction; Human IgG; Gold electrode

1. Introduction

The quartz crystal microbalance (QCM) based on the oscillation/resonance of a piezoelectric quartz crystal (PQC) is a device capable of determining in situ an electrode mass change down to the nanogram level. The detection is based on the Sauerbrey equation \[1\], which displays a linear relationship between the mass change \((\Delta m, \text{ in g})\) and the frequency change \((\Delta f, \text{ in Hz})\) and is valid for loading and removal of a thin, rigid, and homogeneous film both in a liquid solution and in a gaseous environment.

\[
\Delta f = - \frac{2f_0^2}{(\rho_q \mu_q)} \frac{\Delta m}{A} = - 2.264 \times 10^{-6} f_0^2 \frac{\Delta m}{A}
\]

where \(f_0\) in Hz is the resonant frequency of the fundamental mode of the crystal, \(A\) in cm\(^2\) is the piezoelectrically active area, \(\rho_q\) (=2.648 g cm\(^{-3}\)) is the density of quartz, and \(\mu_q\) (=2.947 \times 10^{11} \text{ g cm}^{-1} \text{ s}^{-2})\) is the shear modulus of quartz.

In addition, the PQC frequency is also sensitive to the viscous properties of the loading liquid when the QCM is used in the liquid phase \[2–4\]. A study by Kanazawa and Gordon indicated the effect of the liquid density \((\rho_L)\) and viscosity \((\eta_L)\) on the resonant frequency \((\Delta f_L)\) in the following way \[5\]:

\[
\Delta f_L = - \frac{f_0^{3/2}(\rho_L \eta_L)^{1/2}}{(\pi \rho_Q \mu_Q)^{1/2}}
\]

As one parameter of the Butterworth Van-Dyke (BVD) equivalent circuit for an AT-cut quartz crystal, the motional resistance \((R_1)\) can be expressed by \[6\]:

\[
R_1 = \frac{(2\pi f_0 \rho_L \eta_L)^{1/2} A}{k^2}
\]

where \(k\) is the electromechanical coupling factor. A net liquid-loading effect for PQC with one side contacting solution can be characterized by the following equation \[7\]:

\[
\Delta R_{1L} = - \frac{4\pi L_q \Delta f_L \sqrt{f \mu_Q}}{\sqrt{\bar{c}_{66} f_g}} \approx - 4\pi L_q \Delta f_L
\]

where \(L_q\) is the motional inductance of the quartz crystal in air, \(\bar{c}_{66}\) is the lossy piezoelectrically stiffened quartz elastic constant \(2.957 \times 10^{11} \text{ g cm}^{-1} \text{ s}^{-2}\) \[3,8\]. According to this equation, the characteristic slope value of \(\Delta f/\Delta R_1\) for a net density/viscosity effect on the 9 MHz PQC resonance is \(-\approx 10 \text{ Hz} \text{ Q}^{-1}\). Obviously, the larger
the absolute value of $\Delta f/\Delta R_1$, the weaker the viscous effect and the stronger the mass effect.

Electrochemical impedance spectroscopy (EIS) is a powerful electrochemical method for investigating electrode processes and determining surface adsorption kinetics as well as mass transport parameters through adopting smaller electrochemical perturbations than some transient electrochemical techniques [8,9]. The electrochemical complex impedance ($Z$) can be represented as a sum of the real ($Z_{re}$) and imaginary ($Z_{im}$) components ($Z=Z_{re}+jZ_{im}$, where $j=\sqrt{-1}$) that originate generally from the resistance and capacitance of an electrolytic cell, respectively. While EIS analyses with appropriate equivalent circuits allow one to obtain information of a film modified on an electrode surface, the modification of a chemical or biological substance on an electrode could also be monitored by measuring the electrochemical impedance (EI) at a fixed measurement frequency.

Both EIS and QCM methods have been widely used to investigate surface adsorption and/or immobilization behavior of proteins or other biomaterials that are of fundamental importance in the development of medical devices, biotechnology, and biosensors [8,10,11]. It is obviously expected that an EIS–QCM combination measurement is powerful for its providing sufficient information of interfacial characteristics, including the electrode mass, the viscoelasticity of a foreign film, the local solution density/viscosity property near the electrode surface, and the interfacial capacitance. To the best of our knowledge, this combination method has not been reported for the study of antibody–antigen (Ab–Ag) reaction. In this work, we aim at the comparative study on antihuman IgG (hIgG) adsorption onto bare poly(o-phenylenediamine) (PPD)- and 1-dodecanethiol (C12SH)-modified Au electrodes and human IgG reaction by the EIS–QCM combination system, and both the association constant of the immunoreaction and the valence of human IgG are simultaneously obtained.

2. Materials and methods

2.1. Materials

Goat-antihuman IgG antibody (anti-h IgG), human IgG (hIgG), and rabbit IgG (rIgG) were purchased from Dingguo Biotechnology (Beijing). Reaction buffer was a phosphate-buffered saline (PBS; pH 7.4), where the PBS prepared was a mixture of 8.0 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, 137 mM NaCl, and 2.7 mM KCl. o-Phenylenediamine (OPD) was purchased from Shanghai Chemical Reagent. 1-dodecanethiol (C12SH) was purchased from Sigma and used as received. All other reagents were of analytical grade, and doubly distilled water was used throughout.

2.2. Instrumentation

Experiments were carried out with a CHI660A electrochemical workstation (CH Instruments, USA) and a research quartz crystal microbalance (Maxtek, USA) for the EIS–QCM combination in this work. The research quartz crystal microbalance is able to accurately record the resonant frequency ($f$) and the motional resistance ($R_1$) of the crystal
via its high-performance phase lock oscillator circuit, as given in the operation manual provided by the manufacturer. Furthermore, the good coincidence of the $f$ and $R_1$ responses to the increase of sucrose concentration between those obtained by using the research quartz crystal microbalance and a HP4395A impedance analyzer [12] has been carefully examined in this laboratory. AT-cut 9-MHz gold-coated piezoelectric quartz crystals (12.5 mm in diameter) were used. The gold electrode of a 6.5-mm diameter on one side of the PQC contacted the solution and served as the working electrode, while the other side of the PQC was located in air. The reference electrode was a saturated KCl calomel electrode (SCE), and all potentials are referred to the SCE in this article. A carbon rod served as the counter electrode.

2.3. Procedures

To remove possible surface contamination, the PQC polycrystalline gold electrode was first treated with three drops of nitric acid then subjected to a voltammetric treatment between 0 and 1.5 V vs. SCE in a 0.2 M HClO$_4$ aqueous solution for sufficient cycles until reproducible cyclic voltammograms (CV) were recorded [8,11]. The treated Au working electrode was then rinsed with doubly distilled water and dried with a stream of clear air prior to use.

PPD was grown at a gold electrode in a 0.05 M OPD+0.1 M H$_2$SO$_4$ aqueous solution by applying cyclic potential sweeps (25 mV s$^{-1}$) in the $-0.4$–$0.95$ V vs. SCE range. The amount of surface deposits was controlled by the frequency response, with the film being grown until $\Delta f = -3.3$ kHz. The C12SH-modified gold electrode was prepared by immersing a bare gold electrode in a 5 mM C12SH ethanol solution for 1 h. The prepared electrode was rinsed thoroughly with ethanol and then doubly distilled water.

The procedures for anti-h IgG adsorption and subsequent hIgG reaction experiments are illustrated in Scheme 1. Au electrodes before and after anti-h IgG adsorption (or hIgG reaction) were characterized in a PBS buffer containing 2.00 mM K$_4$Fe(CN)$_6$ via EIS

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**Scheme 1. Experimental steps for anti-h IgG adsorption and subsequent hIgG reaction experiments.**
method and cyclic voltammetry (all at 25 mV s\(^{-1}\)). For EIS measurements in the presence of \(K_4Fe(CN)_6\), the working electrode potential was fixed at the formal potential of the ferricyanide/ferrocyanide couple after being conditioned at this potential for 90 s at first. In each anti-h IgG adsorption (or hIgG reaction) experiment, 60 \(\mu\)L of 1.0 g l\(^{-1}\) anti-h IgG (or hIgG) was speedily injected into a PBS buffer of 0.3 mL to give a final concentration of 0.167 g l\(^{-1}\) anti-h IgG (or hIgG), and simultaneous QCM and EI measurements were conducted to trace the adsorption and reaction processes (generally lasted for ~2000 s). It should be noted that the loss of the adsorbed anti-h IgG during the EIS and CV characterizations in step 4 were negligible here, as experimentally examined from the change of QCM frequency within 5 Hz in PBS solution before and after the characterizations. The final concentration of 0.167 g l\(^{-1}\) anti-h IgG (or hIgG) was selected and fixed for all adsorption experiments for comparison; also, the adsorption of anti-h IgG on a bare Au electrode in the PBS buffer at pH 7.4 could be saturated at this concentration. The experimental procedures for estimation of the association constant of the immunoreaction and the valence of antigen will be given in captions of related figures and text. All experiments were conducted at 20±2 °C.

3. Results and discussion

3.1. Adsorption of anti-h IgG onto bare, PPD- or C12SH-modified electrodes

The QCM data and electrochemical impedance responses during anti-h IgG adsorption onto bare, PPD-, and C12SH-modified electrodes are shown in Fig. 1. The \(R_s\) and \(C_s\) data given here were obtained from EIS analyses using a series \(R_s–C_s\) equivalent circuit of the electrochemical cell [9]. We found that \(f\) decreased always abruptly, while \(\Delta R_1\) increased after the anti-h IgG addition. Meanwhile, \(\Delta R_s\) and \(\Delta C_s\) also decreased. Generally, changes in \(R_s\) should reflect variations of viscous properties of the solution and foreign film as they represent an oscillation energy loss into the surrounding environment [13]. As is well known, an accurate estimation of a thin film in a liquid solution via the Sauerbrey equation requires that the film be sufficiently rigid. For the experiments shown in Fig. 1, the absolute values of \(\Delta f/\Delta R_1\) are greater than 100 Hz \(\Omega^{-1}\), which are obviously larger than the characteristic value of ~10 Hz \(\Omega^{-1}\) for 9 MHz crystals, suggesting that the mass effect predominates the frequency change. Experimentally, when another aliquot of anti-h IgG solution was added to the solution after a 2000-s adsorption, the frequency and resistance changes were very minor (within ±5 Hz for frequency and ±0.5 \(\Omega\) for resistance), implying that the viscous effect induced by the addition of anti-h IgG solution is negligible. Therefore, \(\Delta f\) could be approximately taken as a measure of the mass of adsorbed anti-h IgG via the Sauerbrey equation, and \(f\) vs. time curves reflect adsorption kinetics of anti-h IgG. In addition, \(C_s\) could represent the interfacial capacitance according to a simplified \(R_s–C_s\) equivalent circuit of EIS in the absence of electroactive species [9], and thus it also depicts the adsorption kinetics of anti-h IgG. We found here that the time-dependent responses \((r,\) here \(\Delta f\) and \(\Delta C_s\)) can be well simulated by the following empirical equation [14]:

\[
r(t) = a_0 + a_1\exp(-t/\tau_1) + a_2\exp(-t/\tau_2)
\]

(5)
where $a_0, a_1, a_2, \tau_1$ and $\tau_2$ are constants to be fitted. Since $a_0$ obviously represents $r(t)$ at $t \to \infty$, then it is useful to extract the equilibrium value of a response from data of a time-limited experiment, e.g., in the 1-dodecanethiol-modified case that the resonant frequency somewhat decreased even at 2000 s. We fitted responses of $\Delta f$ and $\Delta C_s$ according to Eq. (5)
using a nonlinear fitting program embedded in the SigmaPlot Graphing Software V2.0. We found that Eq. (5) fits the responses of $\Delta f$ and $\Delta C_s$ very well, as characterized by small values of the relative sum of residual squares defined as

$$q_r = q/\sum_{i=1}^{N} r_{expl}^2 = \sum_{i=1}^{N} \left( r_{fit} - r_{expl} \right)^2 / \sum_{i=1}^{N} r_{expl}^2$$

(6)

where $q_r$ is the sum of residual squares, $r_{fit}$ is the fitted results according to Eq. (5), $r_{expl}$ is the experiments responses, $N$ is the number of data points.

The results after fitting the responses of $\Delta f$ and $\Delta C_s$ given in Fig. 1 are summarized in Table 1. We found that $\Delta f$ and $\Delta C_s$ vs. time curves obtained from the fitting were very close to the experimental ones. By using the Sauerbrey equation, the values of equilibrium surface coverage of anti-h IgG at bare, PPD-, and C12SH-modified Au electrodes, 1.36, 1.42, and 2.08 $\mu$g cm$^{-2}$, can be worked out from corresponding $a_0$ values of best fit for $\Delta f$, $-248$, $-260$, and $-379$ Hz, respectively. This finding indicates that a more hydrophilic Au electrode surface exhibits a stronger tendency against anti-h IgG adsorption. Since the previous PPD or C12SH modification on Au electrodes had led to a large decrease in $C_s$, decreases in $C_s$ were significantly smaller on PPD- and C12SH-modified Au electrodes than those on the bare one. Similar to $\Delta f$, $a_0$ values of best fit for $\Delta C_s$ on bare, PPD-, and C12SH-modified Au electrodes are $-7.68$, $-0.69$, and $-0.07$ $\mu$F, respectively.

### 3.2. Reaction of adsorbed anti-h IgG with hIgG

Fig. 2 shows the time courses of the simultaneous responses of $\Delta f$, $\Delta R_1$, $\Delta R_s$, and $\Delta C_s$ during the reaction of hIgG with anti-h IgG adsorbed on bare, PPD-, and C12SH-modified Au electrodes. Changes in $f$, $R_1$, and $C_s$ after the addition of hIgG were similar to those of the above anti-h IgG adsorption, while $R_s$ increased on PPD- and C12SH-modified Au electrodes. To discriminate the specific reaction, a control experiment via addition of an rIgG solution was carried out, and the corresponding response curves are shown in Fig. 2. The addition of rIgG led to a relatively small decrease in $f$ and no obvious changes in $R_1$ and $R_s$, while decreases in $C_s$ were almost equal to those after hIgG addition. This may be interpreted as follows. Adsorption of rIgG onto anti-h IgG-coated electrodes was nonspecific and random, thus it only led to a slight decrease in $f$ due to little mass loading and variations in solution density/viscosity. However, $C_s$, representing the interfacial capacitance, reflects changes in the dielectric properties of electrode/solution interface. The dielectric changes of the electrode/solution interface induced by the site-

### Table 1

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Response</th>
<th>$a_0$</th>
<th>$a_1$</th>
<th>$a_2$</th>
<th>$\tau_1/s$</th>
<th>$\tau_2/s$</th>
<th>$q_r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare Au</td>
<td>$\Delta f$</td>
<td>$-248$</td>
<td>114</td>
<td>61.7</td>
<td>34.1</td>
<td>1211</td>
<td>$2.67 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>$\Delta C_s$</td>
<td>$-7.68$</td>
<td>7.04</td>
<td>0.68</td>
<td>23.7</td>
<td>653</td>
<td>$1.68 \times 10^{-5}$</td>
</tr>
<tr>
<td>PPD/Au</td>
<td>$\Delta f$</td>
<td>$-260$</td>
<td>185</td>
<td>62.1</td>
<td>36.5</td>
<td>954</td>
<td>$8.12 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>$\Delta C_s$</td>
<td>$-0.69$</td>
<td>41.5</td>
<td>0.73</td>
<td>1.26</td>
<td>1389</td>
<td>$1.25 \times 10^{-3}$</td>
</tr>
<tr>
<td>C12SH/Au</td>
<td>$\Delta f$</td>
<td>$-379$</td>
<td>349</td>
<td>99.8</td>
<td>45.4</td>
<td>660</td>
<td>$2.41 \times 10^{-4}$</td>
</tr>
<tr>
<td></td>
<td>$\Delta C_s$</td>
<td>$-0.07$</td>
<td>0.047</td>
<td>0.036</td>
<td>66.9</td>
<td>833</td>
<td>$1.86 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

$a_0$, $a_1$, and $a_2$ are in Hz for $\Delta f$ and in $\mu$F for $\Delta C_s$, respectively.
selective hIgG combination and random rIgG adsorption may be small, the decreases in $C_s$ were thus almost equal. Similar to the above anti-h IgG adsorption, according to the Sauerbrey equation, $a_0$ values of best fit for $\Delta f$, here $-159$, $-169$, and $-216$ Hz, corresponding to the electrode surface coverage of combined hlgG molecules of 0.871, 0.925, and 1.18 $\mu$g cm$^{-2}$ at bare, PPD-, and C12SH-modified Au electrodes, respectively.

Fig. 2. Time courses of simultaneous response of $\Delta f$, $\Delta R_1$, $\Delta R_s$, and $\Delta C_s$ during hlgG reaction on bare (1), PPD-(2) and C12SH-modified (3) Au electrodes. Curve 0 shows a control experiment for the nonspecific adsorption of rlG onto anti-h IgG-coated Au electrode. Other experimental conditions are the same as those in Fig. 1.
Since both anti-h IgG and hIgG belong to immunoglobulins of the IgG family and have a molecular weight of $1.50 \times 10^5$ [15], according to the saturated $\Delta f$ values for antihuman IgG adsorption and succeeding human IgG combination, the combination ratios of adsorbed anti-h IgG to hIgG could be estimated here as 1:0.64, 1:0.65, and 1:0.56 at bare, PPD-, and C12SH-modified Au electrodes, respectively. It should be noted that the combination ratio here refers to the ratio of the total amount of the antibody adsorbed on the electrode to that of the subsequent associated antigen. The valence of anti-h IgG is 2, which means that an anti-h IgG antibody molecule contains two binding sites which recognize specific areas of the antigen (hIgG) [15]. At immunoreaction equilibrium, the amount of the Ag-occupied sites in antibody must be equal to that of the Ab-occupied sites in antigen. By considering that nearly 50% of the binding sites in the surface-confined anti-h IgG molecules may be blocked by the electrode as a result of their random-oriented adsorption on the electrode surface and the valence of hIgG is 2 (two sites for binding to anti-h IgG), as will be discussed later, the combination ratio of adsorbed anti-h IgG to hIgG is thus expected to be close to or slightly larger than 1:0.5. The acceptable agreements among the three combination ratios may imply that the bioactivity of anti-h IgG on the three different kinds of surface was comparable with one another. The small differences among the combination ratios may be understood as follows. The C12SH-modified gold electrode exhibited the most hydrophobic surface, while the other two

![Fig. 3. Cyclic voltammograms (A) for bare (solid line), anti-h IgG- (dashed line), and hIgG- (dotted line) modified Au electrodes as well as electrochemical impedance spectroscopy (B) for bare ( ), anti-h IgG- (■), and hIgG-modified ( ) Au electrodes in a PBS solution (pH 7.4) containing 2.00 mM K$_4$Fe(CN)$_6$. (A) $dE/dt=25$ mV s$^{-1}$; (B) 100 kHz–0.5 Hz, 10 mV rms, 0.20 V vs. SCE.](image-url)
electrodes had more hydrophilic surfaces. The very strong hydrophobic interaction between protein and the hydrophobic electrode surface resulted in a very large amount of protein adsorption and might induce orientation of adsorbed anti-h IgG molecules somewhat different from those on a hydrophilic surface, leading to the small difference in the combination ratio at the C12SH-modified Au electrode.

3.3. Electrochemical impedance and cyclic voltammetry investigation of adsorption of antibody and antibody–antigen reaction

To examine effects of surface adsorption on the electrode kinetics, we conducted potential cycling and electrochemical impedance experiments before and after anti-hIgG adsorption and hIgG reaction on bare, PPD-, and C12SH-modified Au electrodes, as shown in Figs. 3–5, respectively. Analyses of CV data via the EG&G COOL software readily produced $k_s$ values. $k_s$ values from EIS were obtained from the charge transfer resistance ($R_{ct}$) after EIS data analysis via the Randles equivalent circuit and nonlinear EIS fitting software edited by Boukamp [16]. Since data formats of CV and EIS obtained from CHI660A are different from those of the data analysis software we purchased, in this work, macro programs recorded in Microsoft Word2000/Win98 was thus used for this purpose.

![Cyclic voltammograms (A) for bare (solid line), PPD- (dashed line), anti-h IgG- (dotted line), and hIgG-modified (dash dotted line) Au electrodes as well as electrochemical impedance spectroscopy (B) for bare (▲), PPD- (▲), anti-h IgG- (■), and hIgG-modified (○) Au electrodes in a PBS solution (pH 7.4) containing 2.00 mM K$_3$Fe(CN)$_6$. (A) $dE/dt$=25 mV s$^{-1}$; (B) 100 kHz–0.5 Hz, 10 mV rms, 0.20 V vs. SCE. The Nyquist plot for the bare gold electrode was 5× enlarged for clarity.](image-url)
The results are summarized in Table 2. The anti-h IgG adsorption on the bare gold electrode decreased the redox reversibility of Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$ couple, owing to the barrier effect of adsorbed anti-h IgG and nonconducting anti-h IgG molecules of large size on the access.

### Table 2

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Measurement method</th>
<th>$k_s$ (cm s$^{-1}$)</th>
<th>After PPD or C12SH modification</th>
<th>After anti-h IgG modification</th>
<th>After IgG modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare Au</td>
<td>CV</td>
<td>2.6×10$^{-3}$</td>
<td></td>
<td>2.3×10$^{-6}$</td>
<td>1.4×10$^{-6}$</td>
</tr>
<tr>
<td></td>
<td>EIS</td>
<td>1.1×10$^{-3}$</td>
<td></td>
<td>2.3×10$^{-6}$</td>
<td>1.4×10$^{-6}$</td>
</tr>
<tr>
<td>PPD/Au</td>
<td>CV</td>
<td>2.1×10$^{-3}$</td>
<td>2.5×10$^{-7}$</td>
<td>4.4×10$^{-8}$</td>
<td>2.4×10$^{-8}$</td>
</tr>
<tr>
<td></td>
<td>EIS</td>
<td>0.92×10$^{-3}$</td>
<td></td>
<td>4.4×10$^{-8}$</td>
<td>2.4×10$^{-8}$</td>
</tr>
<tr>
<td>C12SH/Au</td>
<td>CV</td>
<td>2.9×10$^{-3}$</td>
<td>2.5×10$^{-7}$</td>
<td>4.4×10$^{-8}$</td>
<td>2.4×10$^{-8}$</td>
</tr>
<tr>
<td></td>
<td>EIS</td>
<td>1.1×10$^{-3}$</td>
<td>2.8×10$^{-8}$</td>
<td>2.8×10$^{-9}$</td>
<td>2.4×10$^{-9}$</td>
</tr>
</tbody>
</table>

$^a$ CV: cyclic voltammetry; EIS: electrochemical impedance spectroscopy, measured at 10 mV rms, 10 kHz–0.5 Hz, and an electrode-potential of 0.20 V vs. SCE (the formal potential of the Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$ couple in this medium). $k_s$ values from CV and EIS are mean values of two successive measurements with relative deviation values smaller than 6%.
of Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$ species to the Au surface. The modification of the PPD film and the monolayer assembly of 1-dodecanethiol of a long carbon chain greatly decreased $k_s$ due to their great barrier effect on the electron transfer between Au electrode and Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$ species. The adsorption of anti-h IgG and the succeeding combination of hIgG on the PPD- and C12SH-modified Au electrode decreased the redox reversibility further, suggesting the formation of more closely packed surface layers against the electron transfer between the Au electrode and Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$ species.

3.4. Simultaneous estimation of the association constant of the immunoreaction ($K_a$) and the valence of antigen ($s$)

The equation [17,18] used to fit the association curves and estimate association constants is

$$\frac{1}{K_a c_F s} + 1 = \frac{\Delta f^0}{\Delta f}$$

where $K_a$ is the association constant, $c_F$ is the concentration of free antigen in solution, $s$ is the valence of antigen, namely, the amount of the binding sites of the antigen molecule which recognize specific areas of the antibody, $\Delta f$ is the frequency change in hertz measured at each antigen concentration, and $\Delta f^0$ is the frequency at saturation. Since the concentration of associated antigen is far smaller than that of the initial antigen in solution $c_P$, the approximation of $c_F c_F - c_F$ is reasonable. This equation is also named as the Langmuir model since it is similar to the Langmuir adsorption equation. Therefore, Eq. (7) may be transformed to

$$\frac{c_F}{\Delta f} = \frac{1}{K_a \Delta f^0 s} + \frac{c_F}{\Delta f^0}$$

where $h$ is the surface coverage, $C_0$ is the capacitance of the antibody layer, $C_{ads}$ is the capacitance caused by specific adsorption of the antigen, $\Delta C_s$ is the capacitance change measured at each antigen concentration, and $\Delta C_s^0$ is the capacitance at saturation. Combining Eq. (10) with Eq. (11), we can obtain

$$\theta = \frac{\Delta C_s}{\Delta C_s^0}$$

The Langmuir model adsorption is expressed by

$$\theta = \frac{K c_F}{1 + K c_F}$$
According to Eqs. (12) and (13), the Langmuir isotherm equation is rewritten as
\[
\frac{c_F}{\Delta C_s} = \frac{1}{K_a \Delta C_s^0} + \frac{c_F}{\Delta C_s^0} \quad (14)
\]

The combination of Eq. (8) with Eq. (14) yields
\[
\frac{1}{\Delta C_s} = \frac{s \Delta f^0}{\Delta C_s^0 \Delta f} - \frac{s - 1}{\Delta C_s^0} \quad (15)
\]

Parameters of $K_a$, $\Delta f^0$, and $\Delta C_s^0$ of best fit were obtained by the linear regression program embedded in the SigmaPlot Graphing Software V2.0. Fig. 6 shows the time courses of simultaneous responses of $\Delta f$, $\Delta R_1$, $\Delta R_s$, and $\Delta C_s$ during the hIgG reaction with anti-hIgG adsorbed on bare Au electrode. Arrows indicate successive additions of 1 g l$^{-1}$ hIgG PBS solutions (10.0 µl for each addition) to a final concentration of 0.0323 (a), 0.0625 (b), 0.0910 (c), 0.118 (d), 0.143 (e), and 0.167 g l$^{-1}$ (f), respectively.
courses of the simultaneous responses of $\Delta f$, $\Delta R_1$, $\Delta R_s$, and $\Delta C_s$ to anti-hIgG/hIgG reaction on a bare Au electrode during step-by-step hIgG concentration increase. The results show that $c_F/\Delta f$ versus $c_F$, $c_F/\Delta C_s$ versus $c_F$, and $1/\Delta C_s$ versus $1/\Delta f$ possess good linear relationships, respectively. The regression equations are $y=-5.70\times 10^{-3}x-1.59\times 10^{-9}$, $y=-0.596x-3.98\times 10^{-7}$, and $y=247x+0.804$, with linearity correlation coefficients of 0.9979, 0.9924, and 0.9981, respectively. According to the slope and intercept, we simultaneously obtained $s$, 2.34, and $K_a$, $1.54\times 10^6$ M$^{-1}$ for $f$ and $1.50\times 10^6$ M$^{-1}$ for $C_s$, which are in acceptable agreement with those in the literatures [15,19].

4. Conclusion

In this work, we have monitored in situ the anti-h IgG adsorption on bare, poly(o-phenylenediamine)-, and 1-dodecanethiol-modified Au electrodes and reaction of hIgG with adsorbed anti-h IgG in PBS buffers via an EIS–QCM combination method. Comparative experiments revealed that the anti-h IgG adsorption was significantly larger on a hydrophobic (1-dodecanethiol-modified) surface, while the bioactivity of antibody on the three kinds of surfaces was almost identical. To characterize the anti-h IgG adsorption and anti-h IgG/hIgG reaction, electrode standard rate constants ($k_s$) of the Fe(CN)$_6^{3-}$/Fe(CN)$_6^{4-}$ couple was measured before and after anti-h IgG adsorption and hIgG reaction. Meanwhile, two simultaneously obtained parameters, $\Delta f$ and $\Delta C_s$, were used to simultaneously estimate the association constant of the immunoreaction and the valence of antigen for the first time. This electrochemical impedance analysis–QCM method is strongly recommended for wider applications in interface-related biochemical and biophysical studies.

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