***In situ* Monitoring of Lysozyme Adsorption onto Bare and Cysteine- or 1-Octadecanethiol-Modified Au Electrodes Using an Electrochemical Quartz-Crystal Impedance System**

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The adsorption of lysozyme from phosphate-buffered saline (pH 7.4) onto bare and cysteine- or 1-octadecanethiol-modified Au electrodes was investigated *in situ* using an electrochemical quartz-crystal impedance system (EQCIS). The equivalent circuit parameters and resonant frequencies of the piezoelectric quartz-crystal (PQC) resonance, the value of half peak width of the electroacoustic conductance spectrum ($Df_{G1/2}$) as well as the electrical double-layer capacitance were obtained and analyzed. The adsorption kinetics was analyzed with a scheme of two consecutive reactions occurring at the interface. The amount of adsorbed lysozyme on a hydrophobic surface was greater than that on a hydrophilic one, and a more negative potential brought a larger amount of adsorbed lysozyme. The standard heterogeneous rate constants of ferri-/ferrocyanide before and after the lysozyme adsorption were evaluated.

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As a kind of amphiphilic substance, proteins exhibit a strong tendency to adsorb at interfaces. A study of the adsorption of proteins on a hydrophilic or hydrophobic surface and an understanding of the interaction of proteins with the solid surface are important for the development of medical devices, biotechnology and biosensors.¹⁻³

Lysozyme is made up of 129 amino acid residues. There short segments of the chain between residues 5 and 15, 24 and 34, 88 and 96, have the structure of an α -helix; the residues between 41 and 45 and 50 and 54 form pleated sheets, and a hairpin turn occurs at residues 46-49. The remaining polypeptide segments of lysozyme have a coil or loop conformation. It is a globular protein with lytic and bactericidal activity.

Piezoelectric quartz-crystal impedance (PQCI) technology has been used to study the protein and DNA adsorption.^{4,5} Usually, PQCI is analyzed on the basis of a Butterworth-van Dyke (BVD) equivalent electrical circuit, which consists of a motional arm and a static arm in parallel, where the static arm contains only the static capacitance (C_0), and the motional arm contains three elements in series, namely, the motional resistance (R_1), the motional inductance (L_1) and the motional capacitance (C_1), all four equivalent circuit parameters have distinct physical meanings.^{6,7} A modified BVD equivalent electrical circuit was proposed by using $L_1 = L_q + L_L + L_f$, $C_1 = C_q$ and $R_1 = R_q + R_L + R_f$, where C_q , L_q and R_q refer to an unperturbed crystal,⁸⁻¹⁰ R_L and L_L are related to the liquid loading effect, R_f and L_f to the presence of the surface-bound film on the electrode. Besides the equivalent-circuit parameters, three characteristic frequencies of the piezoelectric quartz-

crystal (PQC) resonance as follows are often used:¹¹

$$f_0 = \frac{1}{2\pi\sqrt{L_1 C_1}}, \quad (1)$$

$$\omega_s^2 = (2\pi f_s)^2 = \frac{L_1 - C_0 R_1^2 + \frac{2L_1 C_0}{C_1} - \sqrt{C_0^2 R_1^4 + L_1^2 - 2L_1 C_0 R_1^2 - \frac{4L_1 C_0^2 R_1^2}{C_1}}}{2C_0 L_1^2}, \quad (2)$$

$$\omega_p^2 = (2\pi f_p)^2 = \frac{L_1 - C_0 R_1^2 + \frac{2L_1 C_0}{C_1} + \sqrt{C_0^2 R_1^4 + L_1^2 - 2L_1 C_0 R_1^2 - \frac{4L_1 C_0^2 R_1^2}{C_1}}}{2C_0 L_1^2}, \quad (3)$$

where f_0 is the resonant frequency at which the reactance (X) and susceptance (B) of the motional arm vanish, f_s is the series resonant frequency, *i.e.* the lower frequency where the X and B of the BVD circuit vanish, and f_p is the parallel resonant frequency, *i.e.* the higher frequency where the X and B of the BVD circuit vanish, respectively. Note that Eqs. (1) - (3) are valid for the PQC resonance not only in the gaseous phase, but also in the liquid phase.¹¹ The f_s and f_p equations were obtained by solving an equation that combines the conductance (G) and susceptance (B) equations⁶ of the BVD equivalent circuit with a zero-impedance phase-angle condition, *i.e.*, $\theta_z = \tan^{-1}(X/R) = \tan^{-1}(-B/G) = 0$. It is seen that both f_s and f_p are determined by all four equivalent circuit parameters, but f_0 depends only on L_1 and C_1 .

A combination of the PQCI measurement with the electrochemical technology as a more powerful method than the

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Fig. 1 Schematic representation of the EQCIS used in this work. 1: PQC sealed on one terminal of a glass tube using 704 silicon rubber adhesive; 2: test solution; 3: magnetic stirring bar; 4: glass electrolytic cell; 5: counter electrode; 6: reference electrode with a salt bridge.

conventional electrochemical quartz-crystal microbalance (EQCM) has been used for studying metal and polymer deposition,⁹⁻¹¹ and depletion-layer effects.^{12,13} To the best of our knowledge, lysozyme adsorption onto bare and modified Au electrodes has not been studied by such a combination technique. In this work, an electrochemical quartz-crystal impedance system (EQCIS) combining the PQCI measurement with the electrochemical impedance spectrum (EIS) measurement was thus applied to monitor lysozyme adsorption onto bare and modified gold electrodes.

Experimental

Materials

AT-cut 9 MHz piezoelectric quartz crystals (12.5 mm in diameter) were used. There was a silver electrode having a diameter of 6.0 mm on one side of the PQC and a gold electrode having a diameter of 6.5 mm on the other side of it. The gold electrode was vacuum-evaporated by using an Eiko IB-3 ion coater, and a highly pure gold foil purchased from Hitachi Inc. To remove possible surface contamination, the gold electrode surfaces were treated with $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ (v/v 3:1) for 5 min, rinsed thoroughly with water and then cleaned with acetone- and water-wetted filter paper, successively. Modifications of cysteine and 1-octadecanethiol were achieved by soaking the gold electrodes in a 0.005 mol l^{-1} cysteine aqueous solution and in an acetone solution containing 0.005 mol l^{-1} 1-octadecanethiol for 30 min, respectively. Modifications of lysozyme are given in the figure captions.

Lysozyme (from chicken egg white) was purchased from Sigma and was used as received. Doubly distilled water and freshly prepared solutions were employed throughout. All experiments were carried out at room temperature.

Instruments

As shown in Fig. 1, the EQCIS used in this work included an HP 4395A network/spectrum/impedance analyzer, an EG & G M283 + M1025 electrochemical impedance system and two IBM-compatible computers with Intel cards for data sharing. The conductance (G) and susceptance (B) of the PQC resonance were measured synchronously on the HP 4395A equipped with an HP 43961A impedance test adapter and an HP 16092A test fixture. A user program was written in Visual Basic (VB) 5.0 to control the HP 4395A and to acquire admittance data via an HP 82341C high-performance HP-IB interface card for Windows

Table 1 Values of the contact angle/degree

Bare Au electrode surface	After thiol pretreatment		After lysozyme modification
	Cysteine	1-Octadecanethiol	
58	44	111	48
57			49
56			56

Values of the contact angle of the bare Au electrodes were measured after immersing these electrodes into the background electrolyte for *ca.* 30 min.

3.1/NT/95. The VB program included a SICL4 module from HP and a voice-interpretation module to produce voice message during impedance calibration of the HP 16092A test fixture. Time, number of frequency scans, the series resonant frequency (f_s , *i.e.* the smaller frequency at zero B, found automatically by the HP 4395A with a frequency resolution of 0.001 Hz) and the maximum G value were displayed during measurements. A non-linear least square fitting program based on the Gauss-Newton algorithm was written in VB 5.0 for the simultaneous fits of G and B data.

A saturated KCl calomel electrode (SCE) with a supporting electrolyte salt bridge served as the reference and all potentials in this work were referenced to it. A large-area platinum plate was used as the counter electrode. The gold electrode of the PQC was placed in a test solution, connected to the ground terminal of the HP 16092A test fixture and used as the working electrode, too. The silver electrode of the PQC was placed in air and served only as the connection electrode to the non-ground terminal of the HP 16092A.

Measurements

The measurement conditions of G and B were: 101 points, a frequency span of 40 kHz covering the resonant frequency of the PQC, IF BW of 10 kHz, source power of 0.5 dBm. Prior to admittance measurements, 3-step impedance calibrations of the HP 43961A impedance test adapter (open, short and 50 Ω standard loading) and 2-step compensations of the HP 16092A test fixture (open and short) were carried out. The acquisition period for admittance data was *ca.* 1 s while the HP 4395A completed each frequency scan within the given domain in *ca.* 0.11 s under the selected impedance measurement condition. The EIS measurement under control of EG&G 398 EIS measurement software was conducted simultaneously during the PQCI measurement.

Results and Discussion

The surface hydrophilicity of bare and modified Au electrodes

Anchor of organic molecules on the electrode surface via covalent bonding is an effective approach for modulating the electrode surface hydrophilicity. A cysteine-modified Au electrode surface via S-Au bonding is expected to be relatively hydrophilic because of the presence of many amino and carboxyl groups on its surface, and a 1-octadecanethiol-modified Au electrode via S-Au bonding should be relatively hydrophobic because of the presence of many long-chain alkyl groups on the electrode surface. The values of the contact angle of bare and modified Au electrodes are listed in Table 1. As expected, compared with the contact angle on the bare Au electrode surface, cysteine modification decreased and 1-octadecanethiol modification increased the contact angle,

Fig. 2 Time courses of simultaneous responses of Δf_0 and ΔR_1 during the adsorption of lysozyme onto a 1-octadecanethiol-modified Au electrode in 0.5 mol l⁻¹ NaCl+0.02 mol l⁻¹ NaH₂PO₄+0.08 mol l⁻¹ Na₂HPO₄ aqueous solution (pH 7.4). Arrows indicate additions of lysozyme solutions (1.0 ml) to a final lysozyme concentration of 0.097 g l⁻¹ (1), 0.289 g l⁻¹ (2), 0.447 g l⁻¹ (3), 0.611 g l⁻¹ (4), 0.831 g l⁻¹ (5), 1.244 g l⁻¹ (6), 1.472 g l⁻¹ (7), respectively.

respectively, indicating that cysteine and 1-octadecanethiol molecules were present on the Au electrodes with highly-ordered orientation, respectively.

EQCIS measurements during lysozyme adsorption onto bare and cysteine- or 1-octadecanethiol-modified electrodes

The electrochemical interface can be represented as an electrical circuit composed of the electrolyte resistance (R_e) in series with the parallel combination of the double layer capacitance (C_d) with an impedance (Z_f). For a given frequency (f) which is high enough,^{14,15} the electrochemical interface can be simplified as a series RC circuit ($Z = Z_{re} - jZ_{im}$), and thus R_e and C_d can be calculated from the real part (Z_{re}) and the imaginary part (Z_{im}) of the impedance (Z) according to $R_e = Z_{re}$ and $C_d = 1/(2\pi f Z_{im})$, even if it is not really frequency independent.^{14,15} In this work, the electrochemical impedance was thus measured at 3 kHz.

Figure 2 shows the time courses of the simultaneous responses of Δf_0 and ΔR_1 during the adsorption of lysozyme onto a 1-octadecanethiol-modified Au electrode. Upon the additions of lysozyme solutions, ΔR_1 increased continually and could not reach a quasi-plateau region; and Δf_0 decreased continually and hardly reached a very steady value either. However, the responses of Δf_0 and ΔR_1 did not change rapidly when the lysozyme added was over 0.447 g l⁻¹. The responses of Δf_0 and ΔR_1 were also obtained for lysozyme adsorption onto a bare or cysteine-modified Au electrode, but the changes of R_1 were smaller (<1 Ω), as compared with the case of the 1-octadecanethiol-modified Au electrode, and R_1 also increased rapidly after the final concentration of lysozyme was over ca. 0.65 g l⁻¹. These findings may suggest different adsorption mechanisms on these electrodes, as discussed later. It should be noted that almost no changes in Δf_0 and ΔR_1 were observed when an identical volume of background solution was added, indicating that the above responses of Δf_0 and ΔR_1 were resulted mainly from the adsorption of lysozyme on the electrodes, rather than changes of solution density and viscosity after the lysozyme addition: 0.5 g l⁻¹ was selected as the final concentration of lysozyme in the following lysozyme adsorption experiments.

Figure 3 shows typical responses of ΔR_1 , Δf_0 , $\Delta Df_{G1/2}$, ΔR_e

Fig. 3 Time courses of simultaneous responses of ΔR_1 , Δf_0 , ΔC_d , $\Delta Df_{G1/2}$, ΔR_e and ΔC_d during the adsorption of lysozyme onto bare (1) and cysteine- (2) or 1-octadecanethiol-modified (3) Au electrodes in 0.5 mol l⁻¹ NaCl + 0.02 mol l⁻¹ NaH₂PO₄ + 0.08 mol l⁻¹ Na₂HPO₄ aqueous solution (pH 7.4). 3 kHz, 5 mV rms, 0.1 V vs. SCE. Lysozyme solutions (1.0 ml) were added at time zero to a final concentration of 0.5 g l⁻¹.

and ΔC_d obtained simultaneously during the adsorption of lysozyme onto bare and cysteine- or 1-octadecanethiol-modified Au electrodes, where $Df_{G1/2}$ is the half peak width of G spectrum, as defined later. A +0.1 V vs. SCE bias was applied during electrochemical impedance measurements. It is seen that additions of lysozyme in all cases led to increases in R_1 , $Df_{G1/2}$, and R_e , but to decreases in f_0 . The changes in R_1 were small (within ca. 1 Ω) in the cases of lysozyme adsorption onto either bare or cysteine-modified gold electrode, but the changes in R_1 was larger for the 1-octadecanethiol-modified Au electrode. The motional resistance (R_1) represents the energy dissipation of the PQC resonance into the surrounding environment. The responses of R_1 can, in principle, be understood from increases in the density and viscosity of protein layers adsorbed onto the electrode surface *etc.*, the obvious increases of R_1 observed after adding lysozyme of a 0.5 g l⁻¹ final concentration for the 1-octadecanethiol-modified Au electrode may suggest that many H₂O molecules were simultaneously introduced to the lysozyme surface film during lysozyme adsorption, resulting in an increase in the viscous effect of the surface film because of the hydrophilic increase after the lysozyme adsorption. Protein adsorption onto the surface of the bare or cysteine-modified electrode decreased C_d , as reported previously,¹⁵ but lysozyme adsorption onto the surface of the 1-octadecanethiol-modified Au electrode increased C_d (Figs. 4 and 6). The result may be explained by the increase in the electrode surface hydrophilicity after the adsorption of lysozyme; therefore, more H₂O molecules with a large value of permittivity could accumulate near the electrode surface.

According to the Sauerbrey equation,¹⁶ the mass change can be calculated out from the frequency shift (Δf_0) for the rigid and thin film loading,

$$-\Delta f_0 = 2.26 \times 10^{-6} f_{0g} \Delta m/A, \quad (4)$$

where Δf_0 and f_{0g} are the frequency shift and the fundamental frequency of the quartz crystal in Hz, respectively, Δm is the

Table 2 Parameters obtained by fitting responses of Δf_0 and ΔC_d given in Fig. 3 to Eq. (13)

Au electrode	Response	a_0	a_1	a_2	τ_1/s	τ_2/s	q_r
Bare	$\Delta f_0/\text{Hz}$	-162	44	69	129	1616	7.55×10^{-5}
	$\Delta C_d/\mu\text{F}$	-3.1	0.93	2.3	169	2042	1.05×10^{-4}
Cysteine-modified	$\Delta f_0/\text{Hz}$	-103	31	59	90	1279	1.14×10^{-4}
	$\Delta C_d/\mu\text{F}$	-1.10	0.218	0.866	212	2332	2.35×10^{-4}
1-Octadecanethiol-modified	$\Delta f_0/\text{Hz}$	-424	206	158	88	2178	7.23×10^{-6}
	$\Delta C_d/\mu\text{F}$	0.055	-0.057	-0.087	63	1051	3.67×10^{-4}

a_0, a_1, a_2 , are in Hz for Δf_0 and in μF for ΔC_d , respectively.

mass loaded on the electrode in g, and A is the geometric area of the electrode in cm^2 . In order to estimate the amount of adsorbed lysozyme reasonably from the Δf_0 responses using the Sauerbrey equation, it is certainly very important to evaluate the non-mass effect of lysozyme adsorption on Δf_0 , which should be reflected by the ΔR_1 responses. The relationship between ΔR_1 and Δf_0 due to the net changes in the solution density and viscosity can be obtained from Martin's equations,⁸⁻¹³

$$\Delta R_1 = -\frac{4\pi L_q \Delta f_0 \sqrt{f \mu_q}}{\sqrt{\bar{c}_{66} f_{0g}}} \approx -4\pi L_q \Delta f_0, \quad (5)$$

where L_q and f_{0g} are the motional inductance and resonant frequency of the quartz crystal in air, respectively, μ_q is the shear modulus for the AT-cut quartz ($2.947 \times 10^{10} \text{ N/m}^2$), $\bar{c}_{66}$ is the lossy piezoelectrically stiffened quartz elastic constant ($2.957 \times 10^{10} \text{ N/m}^2$) and f_0 can be approximately used in the calculation instead of f with an error below ca. 0.3%. For the 9 MHz crystal used in this work, the slope of Δf_0 vs. ΔR_1 calculated from Eq. (5) is $10.4 \text{ Hz } \Omega^{-1}$, indicating that the change in liquid density and viscosity equivalent to ΔR_1 of 1Ω is able to cause only about 10 Hz of Δf_0 . If it is reasonable to assume that the viscous effect on Δf_0 is maximum during net changes in the solution density and viscosity (net viscosity effect), the observed ΔR_1 response of ca. 1Ω due to the non-mass (or viscosity) effect of the surface-bound lysozyme layer will thus be expected to cause a Δf_0 response of only several Hz. On the basis of this assumption, since the changes in R_1 were small after the additions of lysozyme solutions for bare and cysteine-modified Au electrodes (within 1Ω), as shown in Fig. 3, Δf_0 should be still a fair approximation for the measurement of the amount of adsorbed lysozyme, regardless of the simultaneous observation of ΔR_1 of ca. 1Ω . In fact, values of the dry mass of the adsorbed lysozyme were found experimentally generally below the wet mass by some 15%, indicating that Sauerbrey's rigid approximation was reasonably valid to estimate the mass of lysozyme adsorbed onto bare and cysteine-modified electrodes under our experimental conditions. But a bigger and continuous increase in R_1 was observed during lysozyme adsorption onto the 1-octadecanethiol-modified electrode in our experiments, indicating the increase in the viscosity of film as explained above. If the viscous effect on frequency shift according to Eq. (5) (ca. -87 Hz) was deducted from the total frequency shift (-424 Hz, shown in Fig. 3), the frequency shift has still -337 Hz, but the dry frequency shift measured was only ca. -220 Hz under our experimental condition. Therefore, it seems reasonable that H_2O molecules plus an electrolyte were introduced into the surface film, leading to the frequency difference.

In addition, the viscous loading on the PQC surface leads to the increase in half peak width of the G curve ($\text{Df}_{\text{G}1/2}$), as show

in Fig. 3. The $\text{Df}_{\text{G}1/2}$, defined as the frequency difference of the two frequency points at half peak height in the G spectrum, can be derived as follows:

$$G_{\text{max}/2} = G_{\text{max}}/2 = 1/(2R_1), \quad (6)$$

According to the admittance (Y) equation of the BVD equivalent circuit model,⁴⁻¹³

$$Y = G + jB = \frac{R_1}{R_1^2 + U^2} + j\left(\omega C_0 - \frac{U}{R_1^2 + U^2}\right), \quad (7)$$

where $\omega = 2\pi f$, $U = \omega L_1 - 1/(\omega C_1) = (1/C_1)(\omega/\omega_0^2 - 1/\omega)$, we obtained

$$G_{\text{max}/2} = \frac{R_1}{R_1^2 + \left(\omega L_1 - \frac{1}{\omega C_1}\right)^2} = \frac{1}{2R_1}, \quad (8)$$

Therefore,

$$\omega_{\text{HG}1/2} = \frac{R_1 C_1 + \sqrt{R_1^2 C_1^2 + 4L_1 C_1}}{2L_1 C_1}, \quad (9)$$

$$\omega_{\text{LG}1/2} = \frac{-R_1 C_1 + \sqrt{R_1^2 C_1^2 + 4L_1 C_1}}{2L_1 C_1}, \quad (10)$$

$$\text{Df}_{\text{G}1/2} = f_{\text{HG}1/2} - f_{\text{LG}1/2} = \frac{\omega_{\text{HG}1/2} - \omega_{\text{LG}1/2}}{2\pi} = \frac{R_1}{2\pi L_1}, \quad (11)$$

where $f_{\text{HG}1/2}$ and $f_{\text{LG}1/2}$ are the higher and lower frequency at half peak height ($G = G_{\text{max}}/2$) in the G spectrum, respectively, and all symbols are in International Unit. $\Delta \text{Df}_{\text{G}1/2}$ also gives similar viscoelastic information to ΔR_1 .

The simplest way to consider the kinetics of lysozyme adsorption is to consider two consecutive reactions occurring at the interface:¹⁵

The second reaction can be associated either with a modification of the structure of the adsorbed molecules in the first layer or with the adsorption of a second layer of proteins upon the first layer. Therefore, the response (r) may be expressed as a sum of two exponential functions,¹⁵

$$r(t) = a_0 + a_1 e^{-t/\tau_1} + a_2 e^{-t/\tau_2}, \quad (13)$$

By taking a_0, a_1, a_2, τ_1 and τ_2 as estimation parameters, we fitted the Δf_0 and ΔC_d responses using the nonlinear fitting

Fig. 4 Time courses of simultaneous responses of Δf_0 and ΔC_d during the adsorption of lysozyme onto the 1-octadecanethiol-modified Au electrode in 0.5 mol l⁻¹ NaCl+0.02 mol l⁻¹ NaH₂PO₄+0.08 mol l⁻¹ Na₂HPO₄ aqueous solution (pH 7.4). 3 kHz, 5 mV rms, 0.1 V vs. SCE. Lysozyme solutions (1.0 ml) were added at time zero to a final concentration of 0.5 g l⁻¹. Solid line: experimental; open circles: fitted according to Eq. (13).

Fig. 5 Time course of simultaneous responses of Δf_0 (solid line), Δf_s (broken line) and Δf_p (dashed line) during adsorption of lysozyme onto cysteine-modified (a), bare (b) and 1-octadecanethiol-modified (c) Au electrodes in 0.5 mol l⁻¹ NaCl + 0.02 mol l⁻¹ NaH₂PO₄+0.08 mol l⁻¹ Na₂HPO₄ aqueous solution (pH 7.4). 3 kHz, 5 mV rms, 0.1 V vs. SCE. Lysozyme solutions (1.0 ml) was added at time zero to a final concentration of 0.5 g l⁻¹.

program embedded in SigmaPlot® Scientific Graphing Software Version 2.0, and the relative sum of residual square was defined as

$$q_r = \frac{\sum_{i=1}^N (r_{\text{fit}} - r_{\text{exp}})^2}{\sum_{i=1}^N r_{\text{exp}}^2}, \quad (14)$$

where r_{fit} and r_{exp} denote the response value fitted and experimentally obtained, respectively, and N is the number of response signal points.

The fitted results of Δf_0 and ΔC_d are well consistent with the experimental results, as shown in Fig. 4. The results after fitting the responses of Δf_0 and ΔC_d given in Fig. 3 are summarized in Table 2. According to the Sauerbrey equation,¹⁶ and the values of a_0 for Δf_0 listed in Table 2, -162, -103 and -424 Hz (using -220 Hz, i.e. the dry frequency shift in the calculation), correspond to the surface coverage of lysozyme of 0.88, 0.56 and 1.20 $\mu\text{g}/\text{cm}^2$ for bare, cysteine- and 1-octadecanethiol-

Fig. 6 Time courses of simultaneous responses of ΔR_1 , Δf_0 and ΔC_d during the adsorption of lysozyme onto 1-octadecanethiol-modified Au electrodes at various potentials -0.2 V (1), 0.1 V (2) and 0.4 V (3) vs. SCE in 0.5 mol l⁻¹ NaCl+0.02 mol l⁻¹ NaH₂PO₄+0.08 mol l⁻¹ Na₂HPO₄ aqueous solution (pH 7.4). 3 kHz, 5 mV rms. Lysozyme solutions (1.0 ml) were added at time zero to a final concentration of 0.5 g l⁻¹.

modified Au electrodes, respectively. Different values of surface coverage of lysozyme should imply different adsorption mechanisms, as given as follows. Protein is made up of charged area, hydrophilic area and hydrophobic area. Therefore, there are three ways that a protein interacts with a solid surface. Proteins are generally anchored on a non-charged surface *via* the interaction of the protein's hydrophobic terminals with hydrophobic surface or the interaction of the protein's hydrophilic terminals with hydrophilic surface. Because a 1-octadecanethiol-modified Au electrode exhibited a hydrophobic surface, lysozyme molecules were anchored on the electrode surface *via* their hydrophobic terminals (alkyl moieties). This interaction was larger than those of hydrophilic terminals of protein with the hydrophilic surface. So the coverage of lysozyme adsorbed on the cysteine-modified Au electrode was smaller than that on the bare and 1-octadecanethiol-modified Au electrodes.

ΔC_0 has been related to the double-layer capacitance of the charged surface.⁴ However, the present results prove that ΔC_0 is not a direct measure of the double-layer capacitance, and may also be determined by other factors which are unclear at present.

The series resonant frequency (f_s) and the parallel resonant frequency (f_p) have been used to analyze protein adsorption. Figure 5 shows responses of f_s and f_p from the experimental results shown in Fig. 3, together with the responses of f_0 as comparisons. f_p is highly sensitive toward the structure of the electrode with the solution interface^{4,17} and f_s is equivalent to the oscillating frequency monitored by a conventional quartz-crystal microbalance driven by an ideal oscillation circuit. Accordingly, if R_1 and C_0 reflect, to some extent, changes in the interfacial properties and the structures of surface-confined species, responses of f_p are expected to be more sensitive to them, too. As expected, the sensitivities of the three frequencies follow the sequence $-\Delta f_p > -\Delta f_0 > -\Delta f_s$, although experimentally and theoretically $f_p > f_s > f_0$. However, the change in each of the equivalent circuit parameters contributes to the responses of Δf_p and Δf_s , and thus analyses of Δf_p and Δf_s are much more complicated compared with those of Δf_0 . Generally, Δf_0 rather than Δf_p and Δf_s , was used to evaluate the mass effect, as theoretically predicted by Martin *et al.*⁸

Fig. 7 Cyclic voltammograms (A) for bare (solid line) and lysozyme-modified (dashed line) Au electrodes as well as electrochemical impedance spectra (B) for bare ($\circ$) and lysozyme-modified ($\square$) Au electrodes in 1 mmol l^{-1} $\text{K}_4\text{Fe}(\text{CN})_6$ +1 mmol l^{-1} $\text{K}_3\text{Fe}(\text{CN})_6$ +0.5 mol l^{-1} NaCl +0.02 mol l^{-1} NaH_2PO_4 +0.08 mol l^{-1} Na_2HPO_4 aqueous solution (pH 7.4). Figure 7A, $dE/dt=20$ mV/s; Figure 7B, 100 kHz – 26.54 mHz, 5 mV rms, 0.215 V vs. SCE. The lysozyme adsorption experiment is shown in Fig. 3.

Fig. 8 Cyclic voltammograms (A) for cysteine-modified (solid line) and cysteine/lysozyme-modified (dashed line) Au electrodes as well as electrochemical impedance spectra (B) for cysteine-modified ($\circ$) and cysteine/lysozyme-modified ($\square$) in 1 mmol l^{-1} $\text{K}_4\text{Fe}(\text{CN})_6$ +1 mmol l^{-1} $\text{K}_3\text{Fe}(\text{CN})_6$ +0.5 mol l^{-1} NaCl +0.02 mol l^{-1} NaH_2PO_4 +0.08 mol l^{-1} Na_2HPO_4 aqueous solution (pH 7.4). Figure 8A, $dE/dt=20$ mV/s; Figure 8B, 100 kHz – 26.54 mHz, 5 mV rms, 0.215 V vs. SCE. The lysozyme adsorption experiment is shown in Fig. 3.

Influences of electrode potentials on the lysozyme adsorption

Lysozyme is positively charged in solutions when the solution pH is lower than its isoelectric point (*ca.* pH 11 for lysozyme). In solutions of pH <11, the cationic lysozyme will be easily adsorbed on negatively charged surfaces. Figure 6 shows responses of ΔR_1 , Δf_0 and ΔC_d obtained simultaneously during adsorption of lysozyme onto 1-octadecanethiol-modified Au electrodes at various potentials. The frequency shift observed at more negative potentials was larger, suggesting a larger amount of adsorbed lysozyme on the 1-octadecanethiol-modified Au electrode at more negative potentials. Generally, the responses of ΔR_1 and ΔC_d were also larger at more negative potentials, which can be understood because the electrode surface hydrophilicity has been increased due to adsorption of hydrophilic lysozyme molecules, and thus the uptake of more water molecules into the surface protein layer, leading to increases in both the viscosity of the surface film and the double layer capacitance.

Influence of adsorption of lysozyme on the electrode kinetics of $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$

Figures 7, 8 and 9 show the results of potential cycling and electrochemical impedance before and after the sequential

Fig. 9 Cyclic voltammograms (A) for 1-octadecanethiol-modified (solid line) and 1-octadecanethiol/lysozyme-modified (dashed line) Au electrodes as well as electrochemical impedance spectra (B) for 1-octadecanethiol-modified ($\circ$) and 1-octadecanethiol/lysozyme-modified ($\square$) Au electrodes in 1 mmol l^{-1} $\text{K}_4\text{Fe}(\text{CN})_6$ +1 mmol l^{-1} $\text{K}_3\text{Fe}(\text{CN})_6$ +0.5 mol l^{-1} NaCl + 0.02 mol l^{-1} NaH_2PO_4 +0.08 mol l^{-1} Na_2HPO_4 aqueous solution (pH 7.4). Figure 9A, $dE/dt=20$ mV/s; Figure 9B, 100 kHz – 26.54 mHz, 5 mV rms, 0.215 V vs. SCE. The lysozyme adsorption experiment is shown in Fig. 3.

Table 3 k_s values of $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$

Au electrodes	$k_s/\text{cm s}^{-1}$
Bare	$^{a}8.8 \times 10^{-3}$, $^{b}8.4 \times 10^{-3}$
Lysozyme-modified	$^{a}3.4 \times 10^{-4}$, $^{b}3.6 \times 10^{-4}$
Cysteine-modified	$^{a}6.4 \times 10^{-3}$, $^{b}6.2 \times 10^{-3}$
Lysozyme/cysteine-modified	$^{a}1.5 \times 10^{-3}$, $^{b}1.6 \times 10^{-3}$
1-Octadecanethiol-modified	$^{a}1.4 \times 10^{-8}$
Lysozyme/1-octadecanethiol-modified	$^{a}1.3 \times 10^{-8}$

a. From cyclic voltammogram. b. From electrochemical impedance spectra (EIS).

surface adsorption of lysozyme on the bare and cysteine- or 1-octadecanethiol-modified Au electrodes. The standard rate constants (k_s) obtained from the EG&G M271 kinetic analyses of the cyclic voltammograms and from the EIS¹⁴ are summarized in Table 3. In all cases, the adsorption of lysozyme decreased k_s , owing obviously to the barrier effects of lysozyme molecules of large size. The k_s value obtained from the 1-octadecanethiol-modified Au electrode is very small, suggesting that a closely-packed 1-octadecanethiol monolayer had formed on the Au surface; and the k_s decreased further after the further modification of lysozyme, demonstrating that lysozyme molecules have interacted with the 1-octadecanethiol self-assembled monolayer to produce a more closely-packed surface layer that blocked the $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ electrode reaction more significantly.

Conclusion

The adsorption processes of lysozyme onto bare and cysteine- or 1-octadecanethiol-modified Au electrodes were monitored simultaneously using a combination of piezoelectric quartz-crystal impedance and electrochemical impedance techniques. Equivalent-circuit parameters, resonant frequencies, the value of half peak width and the electrical double-layer capacitance have been obtained and discussed. The adsorption kinetics has been analyzed by a scheme of two consecutive reactions occurring at

the interface. The standard heterogeneous rate constants of the ferricyanide/ferrocyanide system before and after the adsorption of lysozyme have been estimated from cyclic voltammetry and electrochemical impedance techniques. The electrochemical quartz-crystal impedance technique provides simultaneously not only electrochemical impedance information but also multidimensional piezoelectric information, it is useful for monitoring the adsorption of substances on solid surfaces.

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