Simultaneous Quartz Crystal Microbalance–Electrochemical Impedance Spectroscopy Study on the Adsorption of Anti-human Immunoglobulin G and Its Immunoreaction at Nanomaterial-modified Au Electrode Surfaces

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The quartz crystal microbalance method (QCM), in combination with electrochemical impedance spectroscopy (EIS), has been utilized to monitor *in situ* anti-human IgG adsorption on several Au-based surfaces, bare Au, nanogold/4-aminothiophenol (4AT)/Au, and multi-walled carbon nanotubes (MWCNT)/Au, and succeeding human IgG reactions. Also, the immobilization protocol of anti-human IgG *via* its glutaraldehyde (GA) cross-linking with self-assembled 4AT on an Au electrode and the subsequent surface immunoreaction were examined. The resonant frequency (f_0) and the motional resistance (R_1) of the piezoelectric quartz crystal (PQC) as well as electrochemical impedance parameters were measured and discussed. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) of the ferricyanide/ferrocyanide couple were examined before and after electrode modification, the antibody adsorption and antibody-antigen reactions. We found that the amount for antibody adsorption was the greatest on the colloid Au modified surface, and that at MWCNT ranked the second, while specific bioactivity was almost identical on the four kinds of surfaces. Two parameters simultaneously obtained at the colloid Au modified surface, Δf_0 and ΔC_s (interfacial capacitance), have been used to estimate the association constant of the immunoreaction.

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Introduction

Immunosensors are based on the specificity of antibody-antigen interactions, and a lot of studies have been reported for a wide range of applications in food industry, environmental monitoring, biotechnology, pharmaceutical chemistry and clinical diagnostics in the last two decades.^{1–8} Antigen or antibody immobilization is vital in constructing a biosensor, and the reported immobilization methods are mainly based on the polymer membrane,⁹ Langmuir–Blodgett film,¹⁰ protein A premodification,⁷ or self-assembled monolayer (SAM).¹¹ Among them, the SAM technique offers one of the simplest ways to provide a reproducible, ultrathin, and well-ordered layer suitable for the further modification of antibodies or antigens.

Recently, various nanomaterials, *e.g.* carbon nanotubes and metal/semiconductor nanoparticles, have found numerous applications in biological and chemical fields.^{12–14} Colloidal Au has gained much attention in biological studies due to its potential advantages such as easy preparation, good biocompatibility, and relatively large surface. Some enzymes, such as lactate oxidase and tyrosinase, still retain their bioactivity when adsorbed onto colloidal gold.^{15,16} The current response of glucose oxidase was found to be higher after it was mixed with colloidal Au.¹² It has been suggested that the direct adsorption of proteins onto bulk metal surfaces usually leads to the denaturation of these proteins

and the loss of their bioactivity; however, these proteins will retain their bioactivity while adsorbed onto colloidal Au.¹⁴ Thus, colloidal Au provides a biocompatible environment for protein immobilization. However, the mechanisms for protein absorption on the gold nanoparticles and interaction between proteins remain to be clarified in detail.

The quartz crystal microbalance (QCM) has been widely employed by analysts and electrochemists mainly as a mass-sensitive transducer. The detection is based on the Sauerbrey equation,¹⁷ which displays a linear relationship between the mass change and the frequency change valid for loading and removal of a thin, rigid and uniformly distributed film in the gaseous environment. In the liquid phase, the QCM response is sensitive not only to mass loading but also to changes in the density and viscosity of the solution near the electrode. Kanazawa *et al.* pioneered an equation that depicts the oscillation-frequency response of a piezoelectric quartz crystal (PQC) to the solution density and viscosity.¹⁸ Later, Martin *et al.* derived a series of equations of equivalent circuit parameters for a PQC with simultaneous mass and liquid loading.¹⁹ According to Martin's equations, 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 is calculated to be about $-10 \text{ Hz } \Omega^{-1}$. The PQC-based biosensor has been widely used for analyses of protein,²⁰ nucleic acid,²¹ and enzyme,²² and for immunoassay,²³ *etc.*

Electrochemical impedance spectroscopy (EIS) is a powerful electrochemical method for investigating electrode processes and determining surface-adsorption kinetics as well as mass-

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transport parameters, through adopting smaller electrochemical perturbations than some transient electrochemical techniques.^{24,25} 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.^{24,26,27} It is obviously expected that an EIS-QCM combination measurement will be powerful for 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.

We are interested in making clear the characteristics of protein adsorption on nanomaterials for developing nanoscale and/or nano-enhanced biosensors with sensing performance better than that of conventional ones. Based on several previous reports,²⁸⁻³⁰ in this work, we study anti-human IgG adsorption onto 4-aminothiophenol/nanogold colloids (nanoAu/4AT/Au) and multi-walled carbon nanotubes-modified Au (MWCNT/Au) electrodes, and in sequence human IgG reaction by the EIS-QCM combination method. We include cases of immobilization of anti-human IgG on bare Au and self-assembly of 4-aminothiophenol *via* glutaraldehyde (GA/4AT/Au) cross-linking as comparisons. We found that the amounts for antibody adsorption are greater on the nanomaterials-modified surfaces, while specific bioactivity is almost identical on the 4 kinds of surfaces. The association constant of the anti-human IgG immunoreaction was obtained with satisfactory results.

Experimental

Reagents and chemicals

Goat-anti-human IgG antibody (anti-hIgG), human IgG (hIgG), and rabbit IgG (rIgG) were purchased from Dingguo Biotechnology Co. (Beijing). 4-Aminothiophenol (4AT) was purchased from Sigma. Multi-walled carbon nanotubes (MWCNT) (95% purity, 20–50 nm) obtained from Shenzhen Nanotech. Port. Co. Ltd. (Shenzhen, China) was purified with nitric acid. Reaction buffer was a phosphate buffered saline (PBS, pH 7.4), where the PBS prepared was a mixture of 8.0 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 137 mM NaCl and 2.7 mM KCl. Trisodium citrate, HAuCl_4 , $\text{K}_4\text{Fe}(\text{CN})_6$, $\text{K}_3\text{Fe}(\text{CN})_6$ and all other reagents were commercially available analytical grade reagents, and double-distilled water was used throughout.

Apparatus

The electrochemical piezoelectric quartz crystal impedance system (EQCIS) used here was described previously.²⁷ It allowed fast and simultaneous measurements of the PQC electroacoustic admittance *via* a HP4395A impedance analyzer and the electrochemical impedance *via* a CHI660A electrochemical workstation (CH Instrument Co., USA). A suitable isolation-capacitance ($C_i = 810$ pF) was used to minimize the instrumentation interference on EIS measurements

with CHI660A from simultaneous measurements of the PQCI with HP4395A.²⁷ Synchronous G and B measurements controlled by a user-written Visual Basic (VB) 5.0 program were conducted under conditions of 201 points, a frequency span of 40 kHz covering the PQC resonant frequency. Real-time acquisitions of equivalent circuit parameters at ~ 1 s time intervals were also achieved with the VB 5.0 program through fitting each group of G and B data to a modified BVD model²⁷ based on a Gauss-Newton non-linear least-squares fitting algorithm and a selection of R_1 , f_0 , R_s and C_s as estimation parameters. AT-cut 9 MHz piezoelectric quartz crystals (12.5 mm in diameter) were used experimentally. A home-made three-electrode electrolytic cell of 2 ml volume was used in the experiment. The gold electrode with 6.5-mm diameter on one side of the PQC was exposed to the solution and served as the working electrode; the other side of the PQC faced to air. The reference electrode was a KCl-saturated calomel electrode and a carbon rod was used as the counter electrode. All the potentials reported in this paper are relative to this reference electrode. UV-Vis measurements were performed with a TU-1000 spectrophotometer (Puxi General, China).

Preparation of Au nanoparticles

All pieces of glassware used in the following experiment were cleaned with freshly prepared HNO_3 :HCl (v/v 1:3), rinsed thoroughly with double-distilled water, and dried in air. Au colloid particles were prepared according to reported procedures:³¹ 3.75 ml of 1% sodium citrate solution was added to the boiling aqueous 0.01% chloroauric acid solution, $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (250 ml), and refluxed for about 15 min till a wine-red color was observed. Then the heating source was removed and the colloid was stirred for another 15 min. The UV-Vis spectrum showed that the resulting colloidal solution had an absorption maximum at 520 nm, and transmission electron microscopy (TEM) indicated a particle size of 20 ± 4 nm.³² The solution was stored in dark at 4°C for further use.

Preparation of modified Au electrodes

To remove possible surface contamination, we first treated the PQC polycrystalline gold electrode with three drops of nitric acid, then subjected it to a cyclic voltammetric treatment between 0 and 1.5 V *vs.* SCE in a 0.2 M HClO_4 aqueous solution for sufficient cycles till reproducible cyclic voltammograms (CV) were recorded.^{24,27} The treated Au working electrode was then rinsed with double-distilled water and dried with a stream of clear air prior to use.

The nanoAu/4AT/Au and GA/4AT/Au electrodes were prepared as follows. The self-assembled monolayer of 4AT was formed by placing the bare Au electrode into a freshly prepared 0.1 M 4AT ethanol solution for 4 h at room temperature. After adsorption, the gold electrode modified with 4AT was thoroughly rinsed with absolute ethanol to remove physically absorbed 4AT, then rinsed using lots of double-distilled water, and finally blown dry with high-purity nitrogen. After that, the electrode was immersed in a solution of 2.5 wt% glutaraldehyde (GA) or prepared Au colloid at 4°C overnight. By measuring the dry frequency of the electrode, we found that the frequency shift was about -600 Hz after the Au colloid immobilization.

The MWCNT/Au electrode was prepared as follows: firstly, 1 mg MWCNT and 1 ml H_2O were mixed with ultrasonic agitation over 10 min; secondly, 10 μl MWCNT/ H_2O mixture (optimized volume) was spread evenly onto the electrode surface with a syringe, then dried under an IR lamp. The QCM measurement showed that the frequency shift was about -3000 Hz in air.

Scheme 1 Experimental steps for anti-hIgG adsorption and subsequent hIgG reaction experiments.

QCM and EIS measurements

The procedures for anti-hIgG adsorption and subsequent hIgG reaction experiments are illustrated in Scheme 1. Au electrodes before and after anti-hIgG adsorption (or hIgG reaction) were characterized in a PBS buffer containing 1.00 mM $K_4Fe(CN)_6$ and 1.00 mM $K_3Fe(CN)_6$ via EIS method and cyclic voltammetry (CV) (all at 50 mV s⁻¹). For EIS measurements, the working electrode potential was fixed at the formal potential of the ferricyanide/ferrocyanide couple. In each anti-hIgG adsorption (or hIgG reaction) experiment, 80 μ l of 1.0 g l⁻¹ anti-hIgG (or hIgG) was speedily injected into a PBS buffer of 1.4 ml (under stirring) to give a final concentration of 0.054 g l⁻¹ anti-hIgG (or hIgG), and simultaneous QCM and EI measurements were conducted to trace the adsorption and reaction processes (generally lasting for ~2000 s). It should be noted that the loss of the adsorbed anti-hIgG during the EIS and CV characterizations in step 4 here were negligible, as experimentally examined from the change of the QCM frequency within 5 Hz in PBS solution before and after the characterizations. The final concentration of 0.054 g l⁻¹ anti-hIgG (or hIgG) was selected and fixed for all adsorption experiments for comparison. The experimental procedures for estimation of the association constant of the immunoreaction and the other parameters will be given in captions of related figures and text. All experiments were conducted at room temperature (20 $\pm$ 2 $^{\circ}$ C).

Results and Discussion

Adsorption of anti-hIgG onto the four surfaces

The QCM data and electrochemical impedance responses during anti-hIgG adsorption onto a bare Au, GA/4AT/Au, MWCNT/Au and nanoAu/4AT/Au 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.²⁵ We found that f_0 always decreased abruptly while ΔR_1 increased after the anti-hIgG addition. Meanwhile, ΔR_s and ΔC_s also decreased. Generally, changes in R_1 should reflect variations of viscous properties of the solution and foreign film, as they represent an oscillation-energy loss into the surrounding environment.³⁴ 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 $\Delta f_0/\Delta R_1$ are 50 – 100 Hz Ω^{-1} , which are obviously larger than the

Fig. 1 Time courses of simultaneous responses of Δf_0 , ΔR_1 , ΔR_s and ΔC_s during the anti-hIgG adsorption onto bare Au (a), GA/4AT/Au (b), MCNT/Au (c) and nanoAu/4AT/Au (d) electrodes in PBS buffers (pH 7.4). The final concentration of anti-hIgG was 0.054 g l⁻¹. Electrochemical impedance measurements: 1 kHz, 10 mV rms, and at open circuit potential.

characteristic value of ~10 Hz Ω^{-1} for 9 MHz crystals, suggesting that the mass effect dominates the frequency change. Also, we compared the “wet” and “dry” frequency shifts after the adsorption, and found that the “dry” frequency shifts obtained in air were roughly 68 $\pm$ 4% of the “wet” ones in the liquid. The differences of the “wet” from the “dry” frequency shifts may result from the concomitant oscillation of the electrolyte entrapped into the thin film, and the viscous property of the film may be very small since the $\Delta f_0/\Delta R_1$ is not large. Therefore, the above average ratio of the “dry” over “wet” frequency shift ($r = 0.68$) will be used to calculate the net mass of immobilized proteins. Experimentally, when another aliquot of anti-hIgG solution was added to the solution after 2000-s adsorption, the frequency and resistance changes were very minor (within ± 5 Hz for frequency and $\pm 0.5 \Omega$ for resistance), implying that the viscous effect induced by the addition of anti-hIgG solution is negligible. Therefore, Δf_0 could be approximately taken as a measure of the mass of adsorbed anti-hIgG via the Sauerbrey equation, and Δf_0 vs. time curves reflect adsorption kinetics of anti-hIgG. 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,²⁶ and thus it also depicts the adsorption kinetics of anti-hIgG. We found here that the time-dependent responses (r , here Δf_0 and ΔC_s) can be well simulated by the following empirical equation:³⁵

$$r(t) = a_0 + a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) \quad (1)$$

where a_0 , a_1 , a_2 , τ_1 and τ_2 are constants to be fitted. Since a_0 obviously represents $r(t)$ at $t \rightarrow \infty$, it is useful to extract the

Table 1 Parameters obtained by fitting responses of Δf and ΔC_s given in Fig. 1 according to Eq. (1)

Electrode	Response	a_0	a_1	a_2	τ_1/s	τ_2/s	q_r
Bare Au	Δf_0	-267	82.4	183	98	1036	9.31×10^{-4}
	ΔC_s	-0.85	0.38	0.63	234	924	3.82×10^{-3}
GA/4AT/Au	Δf_0	-293	176	112	245	746	3.58×10^{-3}
	ΔC_s	-0.097	0.064	0.025	379	1022	5.33×10^{-3}
MWCNT/Au	Δf_0	-364	289	57.5	158	967	6.35×10^{-3}
	ΔC_s	-1.05	0.95	0.44	331	1204	1.26×10^{-3}
NanoAu/4AT/Au	Δf_0	-476	396	79.9	71.3	1145	5.29×10^{-4}
	ΔC_s	-1.79	1.41	0.39	184	1325	2.42×10^{-3}

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

Note that 4-s mixing time after each sample addition is corrected during the fitting.

equilibrium value of a response from data of a time-limited experiment, *e.g.* in the colloid Au-modified case that the resonant frequency somewhat decreased even at 2000 s. We fitted responses of Δf_0 and ΔC_s according to Eq. (1) using a non-linear fitting program embedded in the SigmaPlot Graphing Software V2.0. We found that Eq. (1) fits the responses of Δf_0 and ΔC_s very well, as characterized by small values of the relative sum of residual squares defined as

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

where q_r is the sum of residual squares, r_{fit} the result fitted according to Eq. (1), r_{expl} the experiment response, and N the number of data points.

The results after fitting the responses of Δf_0 and ΔC_s given in Fig. 1 are summarized in Table 1. We found that Δf_0 and ΔC_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-hIgG at bare Au, GA/4AT/Au, MWCNT/Au and nanoAu/4AT/Au electrodes, 0.993, 1.09, 1.35 and 1.78 $\mu\text{g cm}^{-2}$, can be worked out from corresponding a_0 values of best fit for Δf_0 , -267, -293, -364 and -476 Hz, respectively. For the QCM used in our experiments, the surface roughness of the bare Au gold electrode measured *via* the reduction charge of the gold oxides in 0.2 mol l⁻¹ aqueous HClO₄ was about 3.6.³⁶ If each anti-hIgG has dimensions of approximately $14 \times 10 \times 5$ nm,³⁷ there will be two possible limits for the area occupied per anti-hIgG on a solid surface: a lower limit of 50 nm² for anti-hIgG adsorbed in a "face-on" orientation, and an upper limit of 140 nm² for an "end-on" orientation of anti-hIgG.³⁸ These two orientations correspond to theoretical frequency changes of 175 and 350 Hz for a protein monolayer, respectively. Our results indicate that adsorptions of both "end-on" and "face-on" orientations of the antibody occurred on the bare Au surface. A rough calculation of the molar ratio of the "end-on" orientation (x) indicated $x = 54\%$ *via* $-350x - 175(1 - x) = -267$, and the approximately 50% value obtained implies that the protein adsorption here is random. Additionally, nanomaterials (especially colloid Au)-modified Au electrode surfaces exhibit a stronger tendency for anti-hIgG adsorption, on which the amount of anti-hIgG was almost double that on bare Au. One reason must be that the nanomaterial-modified electrodes have a larger surface area, which enable antibodies to be bound with higher loading density and antigen binding activity. The major reason for the increase of anti-hIgG adsorption amount is probably an

Fig. 2 Time courses of simultaneous response of Δf_0 , ΔR_1 , ΔR_s and ΔC_s during hIgG reaction on bare Au (a), GA/4AT/Au (b), MCNT/Au (c) and nanoAu/4AT/Au (d) electrodes. Curve r shows a control experiment for the nonspecific adsorption of rIgG onto anti-hIgG/Au electrode. Other experimental conditions are the same as those in Fig. 1.

electrode surface enlargement.³⁰ Meanwhile, due to special characteristics of colloid Au or MWCNT, the modification on Au electrodes led to a larger decrease in C_s than those on the bare Au electrode. However, the decrease in C_s was significantly smaller on the GA/4AT/Au electrode, which may be due to the cross-linking reaction occurring here and the interfacial properties changing little, though the exact mechanism is unknown at present. Similar to Δf_0 , a_0 values of best fit for ΔC_s on nanoAu/4AT/Au, MWCNT/Au, bare Au and GA/4AT/Au electrodes are -1.79, -1.05, -0.85, -0.097 μF , respectively. Also, we note that generally the frequency responses were faster than the ΔC_s , namely, the τ_1 and τ_2 parameters for the frequency response are smaller than those for the ΔC_s , as listed in Tables 1 and 2. This is probably due to the fact that, once a protein molecule was attached to the electrode surface, the frequency response occurred and is hardly expected to change obviously during its slow rearrangement; however, the response of ΔC_s would continue even during the slow rearrangement that causes significant changes in the double-layer structure.

Reaction of adsorbed anti-hIgG with hIgG

Figure 2 shows the time courses of the simultaneous responses of Δf_0 , ΔR_1 , ΔR_s and ΔC_s during the reaction of hIgG with anti-hIgG adsorbed on bare Au, GA/4AT/Au, MWCNT/Au and nanoAu/4AT/Au electrodes. The fitted results for Δf_0 and ΔC_s are summarized in Table 2. Changes in f_0 , R_1 , R_s and C_s after the addition of hIgG were similar to those of the above anti-hIgG adsorption. In order to discriminate the specific reaction, we carried out a control experiment *via* addition of a rIgG solution. The corresponding response curves are shown in Fig. 2. The addition of rIgG led to a relatively small decrease in f_0 and no obvious changes in R_1 , and decreases in C_s and R_s show

Table 2 Parameters obtained by fitting responses of Δf and ΔC_s given in Fig. 2 according to Eq. (1)

Electrode	Response	a_0	a_1	a_2	τ_1/s	τ_2/s	q_f
Bare Au	Δf_0	-168	79	101	123	873	6.17×10^{-4}
	ΔC_s	-0.689	0.63	0.38	247	1120	2.87×10^{-3}
GA/4AT/Au	Δf_0	-157	93	57.2	149	802	3.05×10^{-3}
	ΔC_s	-0.465	31.2	1.59	303	956	2.35×10^{-3}
MWCNT/Au	Δf_0	-189	81	113	158	724	6.95×10^{-3}
	ΔC_s	-0.927	0.74	0.17	261	1237	2.63×10^{-3}
NanoAu/Au	Δf_0	-247	192	56.8	83	1040	1.27×10^{-4}
	ΔC_s	-1.24	1.04	0.09	284	1463	3.62×10^{-3}

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

Note that 4-s mixing time after each sample addition is corrected during the fitting.

relative large values but are also smaller than those after hIgG addition. This may be interpreted as follows. The rIgG adsorption is nonspecific, and thus it may adsorb on the “pinholes”/interspaces of the anti-hIgG layer, leading to a very small frequency change but significant changes in C_s and R_s . The dielectric changes of the electrode/solution interface induced by the small and random rIgG adsorption were also smaller than those induced by the site-selective hIgG combination, and the decreases in C_s were thus small. Similar to the above anti-hIgG adsorption, according to the Sauerbrey equation, a_0 values of best fit for Δf_0 , here -168, -157, -189 and -247 Hz, correspond to the electrode-surface coverage of combined hIgG molecules of 0.626, 0.584, 0.700 and 0.918 $\mu g\ cm^{-2}$ at bare Au, GA/4AT/Au, MWCNT/Au and nanoAu/4AT/Au electrodes, respectively. Since both anti-hIgG and hIgG belong to immunoglobulins of the IgG family and have a molecular weight of 1.50×10^5 ,³⁹ according to the saturated Δf_0 values for anti-hIgG adsorption and succeeding hIgG combination, the combination ratios of adsorbed anti-hIgG to hIgG could be estimated here as 1:0.62, 1:0.53, 1:0.52 and 1:0.52 at bare Au, GA/4AT/Au, MWCNT/Au and nanoAu/4AT/Au electrodes, respectively. 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-hIgG is 2, which means that an anti-hIgG antibody molecule contains 2 binding sites that recognize specific areas of the antigen (hIgG).³⁹ 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-hIgG 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-hIgG), as discussed later, the combination ratio of adsorbed anti-hIgG to hIgG is thus expected to be close to or slightly larger than 1:0.5. The acceptable agreements among the four combination ratios may imply that the bioactivity values of anti-hIgG on the 4 different kinds of surface were comparable with one another. The small differences among the combination ratios may be understood as follows. The MWCNT/Au and nanoAu/4AT/Au electrodes exhibited relatively larger surfaces than the other two electrodes. The larger electrode surfaces with numerous nanoparticles of nanoscale surface curvature resulted in a very large amount of protein adsorption, and might induce orientation of adsorbed anti-hIgG molecules somewhat differently from those on a bare Au surface, leading to the small difference in the combination

Fig. 3 Cyclic voltammograms (A) for bare Au (a), anti-hIgG/Au (b) and hIgG/anti-hIgG/Au (c) electrodes as well as electrochemical impedance spectroscopy results (B) for bare Au (a), anti-hIgG/Au (b) and hIgG/anti-hIgG/Au (c) electrodes in a PBS solution (pH 7.4) containing 1.00 mM $K_4Fe(CN)_6$ + 1.00 mM $K_3Fe(CN)_6$. (A) $dE/dt = 50\ mV\ s^{-1}$; (B) 100 kHz - 0.1 Hz, 10 mV rms, 0.20 V vs. SCE.

Fig. 4 Cyclic voltammograms (A) for bare Au (a), 4AT/Au (b), GA/4AT/Au (c), anti-hIgG/GA/4AT/Au (d) and hIgG/anti-hIgG/GA/4AT/Au (e) electrodes as well as electrochemical impedance spectroscopy results (B) for bare Au (a), 4AT/Au (b), GA/4AT/Au (c), anti-hIgG/GA/4AT/Au (d) and hIgG/anti-hIgG/GA/4AT/Au (e) electrodes in a PBS solution (pH 7.4) containing 1.00 mM $K_4Fe(CN)_6$ + 1.00 mM $K_3Fe(CN)_6$. (A) $dE/dt = 50\ mV\ s^{-1}$; (B) 100 kHz - 0.1 Hz, 10 mV rms, 0.20 V vs. SCE.

ratio at the nanomaterials-modified Au electrode.

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 Au, GA/4AT/Au, MWCNT/Au and nanoAu/4AT/Au electrodes, as shown in Figs. 3–6, respectively. As can be seen from Fig. 3A, the bare Au electrode (curve a) gives good reversibility with a peak-to-peak

Fig. 5 Cyclic voltammograms (A) for bare Au (a), MWCNT/Au (b), anti-hIgG/MWCNT/Au (c) and hIgG/anti-hIgG/MWCNT/Au (d) electrodes as well as electrochemical impedance spectra (B) for bare Au (a), MWCNT/Au (b), anti-hIgG/MWCNT/Au (c) and hIgG/anti-hIgG/MWCNT/Au (d) electrodes in a PBS solution (pH 7.4) containing 1.00 mM $K_4Fe(CN)_6$ + 1.00 mM $K_3Fe(CN)_6$. (A) $dE/dt = 50 \text{ mV s}^{-1}$; (B) 100 kHz – 0.1 Hz, 10 mV rms, 0.20 V vs. SCE.

Fig. 7 Time courses of simultaneous responses of Δf_0 (A), related step-frequency response (B) and step-mass response (C) versus hIgG concentration during the hIgG reaction with anti-hIgG adsorption on a colloid Au modified Au electrode. Arrows indicate successive additions of 1 mg ml^{-1} hIgG PBS solutions (10.0 μl for each addition) to a final concentration of 7.09 (a), 14.1 (b), 21.0 (c), 27.8 (d), 34.9 (e), 41.1 (f), 47.6 (g), and 54.1 $\mu\text{g ml}^{-1}$ (h), respectively.

Fig. 6 Cyclic voltammograms (A) for bare Au (a), 4AT/Au (b), nanoAu/4AT/Au (c), anti-hIgG/nanoAu/4AT/Au (d) and hIgG/anti-hIgG/nanoAu/4AT/Au (e) electrodes as well as electrochemical impedance spectroscopy results (B) for bare Au (a), 4AT/Au (b), nanoAu/4AT/Au (c), anti-hIgG/nanoAu/4AT/Au (d) and hIgG/anti-hIgG/nanoAu/4AT/Au (e) electrodes in a PBS solution (pH 7.4) containing 1.00 mM $K_4Fe(CN)_6$ + 1.00 mM $K_3Fe(CN)_6$. (A) $dE/dt = 50 \text{ mV s}^{-1}$; (B) 100 kHz – 0.1 Hz, 10 mV rms, 0.20 V vs. SCE.

separation within 80 mV, while the anti-hIgG modified electrode (curve b) exhibits an irreversible response. This can be explained by the formation of the anti-hIgG layer on the gold electrode. Figures 4A and 6A (curve b) show the cyclic voltammogram for 4AT modified electrodes. Obviously, the anodic peak currents seriously decreased after electrode surfaces were covered by insulated layers. Figure 6A (curve c) shows the gold nanoparticles-modified electrode, where the anodic

peak current reverts to a slightly smaller value than that of the bare Au electrode. It is reasonable to think that gold nanoparticles will not retard but rather enhance the rate of electrode transfer. Figures 3–6B exhibit the impedance spectroscopy of different modified surfaces of electrodes. The values of Nyquist-circle diameter are summarized in Table 3. The results showed that the anti-hIgG adsorption on the bare Au electrode and succeeding immunoreaction with antigen increased the diameter of Nyquist circles, in other words, decreased the redox reversibility of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ couple, owing to the barrier effect of adsorbed anti-hIgG and non-conducting anti-hIgG molecules of large size on the access of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ species to the Au surface. The modification of the colloid Au on the Au electrode diminished the barrier effect on the electron transfer between Au electrode and $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ species, which enhances the adsorption of anti-hIgG and the succeeding combination amount of hIgG on the colloid Au modified Au electrodes surface. After anti-hIgG adsorption and hIgG reaction, the R_{ct} at the nanoAu/4AT/Au electrode increased more substantially than that at the bare Au, also indicating that more proteins were attached to the surface and thus blocked more effectively the electron transfer.

Thermodynamic study the immobilization of antibody and estimation of the association constant of the immunoreaction (K_a)

The thermodynamic immobilization of anti-hIgG on nanoAu/4AT/Au electrode and antibody-antigen immunoreaction were studied through the real-time output of QCM measurements. The frequency responses in the phosphate buffer to the successive increases in hIgG concentration were recorded for immunoreaction tests.

The equation^{40,41} used to fit the association curves for estimation of association constants is

$$\frac{1}{K_a C_{FS}} + 1 = \frac{\Delta f_0^0}{\Delta f_0} \quad (3)$$

Table 3 AC impedance results for the electrodes obtained from Figs. 3 – 6

Experimental condition	$R_{ct}/k\Omega$				
	Measurement method	Bare Au	After modification	After anti-hIgG adsorption	After IgG adsorption
Bare Au	EIS	0.15		1.6	2.5
GA/4AT/Au	EIS	0.13	5.5	14	17
MWCNT/Au	EIS	0.1	1	1.7	2.3
NanoAu/4AT/Au	EIS	0.1	0.5	9	12

R_{ct} , charge transfer resistance; EIS: electrochemical impedance spectroscopy, measured at 10 mV rms, 100 kHz – 0.1 Hz, and an electrode-potential of 0.20 V vs. SCE (the formal potential of the $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ couple in a PBS solution containing 137 mM NaCl, 2.7 mM KCl, 1.00 mM $\text{K}_4\text{Fe}(\text{CN})_6$ + 1.00 mM $\text{K}_3\text{Fe}(\text{CN})_6$).

where K_a is the association constant, c_F is the concentration of free antigen in solution, s is the valence of antigen (2 for hIgG),⁴² Δf_0 is the frequency change in hertz measured at each antigen concentration, and Δf_0^0 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 $c_F \approx c_P$ is reasonable. This equation is also known as the Langmuir model, since it is similar to the Langmuir adsorption equation. Therefore, Eq. (3) may be transformed to

$$\frac{c_F}{\Delta f_0} = \frac{1}{K_a \Delta f_0^0 s} + \frac{c_F}{\Delta f_0^0} \quad (4)$$

Figure 7(A) shows the time course of Δf_0 and related step-frequency (B) and step-mass (C) responses versus hIgG concentration during step-by-step hIgG concentration increase. A linear regression of $c_F/\Delta f_0$ vs. c_F yields this equation: $y = 2.64 \times 10^{-3}x + 6.28 \times 10^{-9}$ and a linearity correlation coefficient of 0.9993. According to the slope and intercept, we obtain Δf_0 , 262 Hz, and K_a , $3.05 \times 10^6 \text{ M}^{-1}$, which agree with the reported value K_a , $1.5 \times 10^6 \text{ M}^{-1}$ in order of magnitude.^{39,42,43}

Conclusions

In this work we have monitored *in situ* the anti-hIgG adsorption on bare Au, GA/4AT/Au, MWCNT/Au and nanoAu/4AT/Au electrodes and reaction of hIgG with adsorbed anti-hIgG in PBS buffers via an EIS-QCM combination method. Comparative experiments revealed that the anti-hIgG adsorption was significantly larger on nanomaterial-(colloid Au-modified) surfaces while the bioactivity of antibodies on the four kinds of surfaces was almost identical. One reason must be that the nanomaterial-modified electrodes have a relative larger surface area, which enable antibodies to be bound with higher loading density and antigen binding activity. To characterize the anti-hIgG adsorption and anti-hIgG/hIgG reaction, we measured CV and EIS of the $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ couple before and after anti-hIgG adsorption and hIgG reaction. The results show that colloid Au diminished the barrier effect on the electron transfer between Au electrode and $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ species. Meanwhile, both Δf_0 and ΔC_s parameters were simultaneously obtained, and the adsorption of antibody on different electrodes was thermodynamically analyzed. Additionally, the association constant of the immunoreaction was estimated with satisfactory results. The simultaneous QCM-EIS method as a multiparameter and dynamic one may find wider applications in interfacial nano-biochemistry studies.

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