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Original Paper

An electrochemical impedimetric arrayed immunosensor based on indium tin oxide electrodes and silver-enhanced gold nanoparticles

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Abstract. A novel sensitive electrochemical impedance immunoassay based on metal nanoparticle labels and ITO electrodes has been developed. First, 2-aminobenzoic acid (2-ABA) was electropolymerized onto an indium tin oxide (ITO) electrode. The coupling reagents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysulfosuccinimide were then used to activate the electroconductive polymer to form an active ester layer which could react with amino groups of antigen. Subsequently, immunoreaction was carried out between antigen and antibody labeled with gold nanoparticles, followed by the addition of the silver enhancer solution. The charge transfer processes of $[Fe(CN)_6]^{4-}$/$[Fe(CN)_6]^{3-}$ on the ITO surface were affected due to the formation of silver precipitation on the gold nanoparticles, which was determined by electrochemical impedance spectroscopy. The surface was characterized by scanning electron microscopy. Finally, a multiplexed arrayed immunosensor was is described and the samples of antibody and antibody mixture were assayed specifically. The experimental conditions such as the number of electropolymerization cycles and the time of silver enhancement were examined and optimized. The detection range of antibody labeled with gold nanoparticles was between 10.0 ng mL^{-1} and 10.0 μg mL^{-1}.

Keywords: Electrochemical immunosensor; ITO arrayed electrodes; silver enhancement; electrochemical impedance spectroscopy

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Indium tin oxide (ITO) is a well-known electrode material, acting as an optically transparent electrode, which has been employed extensively in spectroelectrochemistry due to its unique optical properties and potential application [1–3]. Moreover, ITO has a wide potential window and possesses stable electrochemical and physical properties, which allows it to be a very promising material for the characterization of biological systems [4–6]. Similar to metal and other metal oxide surfaces, ITO has been modified with a variety of organic layers such as phosphonates [7], silanes [8], amines [9], carboxylic acids [10], thiols [11] and conducting polymers [12] for the immobilization of biomolecules. Numerous studies have been conducted on the development of ITO electrodes and their application to optical sensors [13], direct electron transfer of proteins [14], electrochemical nucleic

acid biosensors [15], electrochemical immunosensors [16], microfluidic on-chip detection [17], and electrochemiluminescence analysis [18]. Among these studies, the electrochemical immunosensors have attracted increasing interest recently owning to their unique advantages such as rapidity, simplicity, inexpensive instrumentation, and field-portability.

The electrochemical immunosensors [19–21], based on the specificity of antigen-antibody interactions with electrochemical transduction, were usually followed by the measurement of the change in potential, current, capacitance or conductivity changes due to the formation of immunocomplex (for example antigen-antibody complex) on the electrode surface. Among these, electrochemical impedance spectroscopy (EIS) has been proved to be a sensitive and effective method to probe the interfacial properties of modified electrode [22–27]. Impedance spectroscopy can be described by a simple equivalent circuit model consisting of resistance and capacitance elements, such as Rs (the solution resistance), Rct (the charge-transfer resistance), CPE (the constant phase element), and a mass transfer element W (Warburg impedance) [28–31]. Our laboratory has also reported impedimetric detection of gold arrayed electrodes modified with aptamers [32] and antibodies [33, 34], by which proteins could be assayed based on label-free [32, 33] or enzyme-labeled amplification approaches [34]. Compared to traditional immunological sensors, arrayed electrodes can be used to assay multiplex targets and this lead to high performance and more information of the detection. Thus, it would be of significance to explore possibility of fabrication of arrayed electrodes by using new materials such as ITO due to its mass production and cost-effectiveness.

In this work, we fabricated a patterned ITO arrayed electrode which independently works both as a working electrode and as a specifically functionalized area. Based on the immunological reactions in these areas, a large amount of silver deposition could be produced specifically on the gold nanoparticle tag and resulted in the amplification of impedance signal and the increase of detection sensitivity. The electrodes exhibit low background signals because of their low electrocatalytic activity and low capacitive current, thus the sensitivity of the electrochemical immunosensors could be improved. The resulting ITO arrayed immunosensor has successfully been used to assay a model antibody mixture consisting of gold-labeled goat anti-hIgG and goat anti-mIgG.

Experimental

Materials

Human IgG (hIgG), gold-labeled goat anti-human IgG (anti-hIgG), gold-labeled goat anti-mouse IgG (anti-mIgG), and mouse IgG (mIgG) were purchased from Beijing Bioting-tech biotechnology Co. Ltd (Beijing, China). 2-aminobenzoic acid (2-ABA) and ethanolamine were obtained from Beijing Institute of Radiation Medicine (Beijing, China). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysulfosuccinimide (NHS), and the silver enhancer solution were obtained from Sigma-Aldrich (Germany). Other chemicals were of analytical reagent grade. All samples and buffers were prepared using deionized water obtained from a Milli-Q water purification system.

Apparatus

Electrochemical measurements were performed on a CHI660 Electrochemical Workstation (CHI Instrumental Inc., Austin, USA). A two-electrode system including the ITO working electrode and a standard Ag/AgCl both as the reference and the counter electrode was employed. The SEM images were taken with a scanning electron microscope (S-3000N, Hitachi).

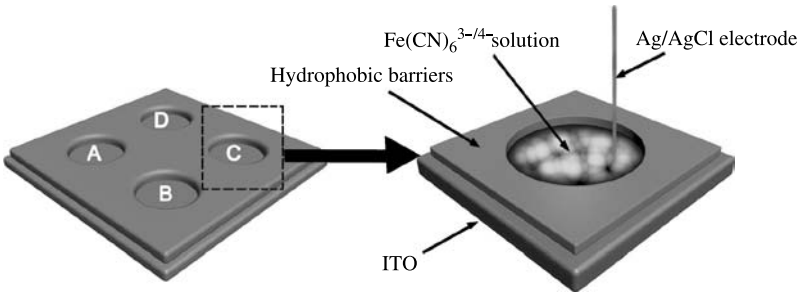
ITO electrode modification

The indium tin oxide (ITO)-coated (Delta Technologies, Stillwater, MN) glass was first patterned to produce four circular working electrodes (diameter of 4.0 mm). Then, they were cleaned according to a procedure reported elsewhere [38]. They were previously sonicated in acetone for 15 min, followed by rinsing with water, ultrasonic agitation in concentrated NaOH in 1:1 V/V water/ethanol, rinsing with deionized water, immersing in $CHCl_3$ for 10 min, and drying.

The modification of ITO electrodes with electroconducting polymer (poly-aminobenzoic acid, PABA) was performed according to the literature [39]. Briefly, the cleaned ITO electrode was first immersed in a solution containing 0.05 M 2-ABA/1 M H_2SO_4, followed by running a CV between 0 V and + 1.0 V for 8 cycles at a scan rate of 40 mV sec^{-1}. The PABA-coated electrode was rinsed with water to remove any nonspecifically adsorbed materials.

Human IgG immobilization and antigen-antibody reactions

The immobilization method via the formation of a covalent bond has been reported previously [15]. In brief, the carboxylic acid groups of the PABA film were further activated by covering the ITO surface with 10 μL of 4 mM EDC/1 mM NHS in 10 mM PBS (pH 7.6) and incubated at room temperature for 1 h, followed by a rinse with deionized water. After that, 4 μL of hIgG (1 mg mL^{-1}) was immediately dropped onto the ITO surface and dried at 4 °C overnight. The IgG-modified ITO electrode was then rinsed throughly with 10 mM PBS (pH 7.6) to remove the weakly adsorbed protein and subsequently incubated in an ethanolamine (0.1 M) solution for 1 h. The electrode was rinsed three times with PBS and then it was exposed to different concentration of gold-labeled goat anti-hIgG in 10 mM PBS (pH 7.6) at room temperature for 2 h, rinsed with PBS and water to remove unbound antibodies before impedance measurements. Thus, the proposed immunosensor was formed, and was stored at 4 °C when not in use.



Scheme 1. The ITO arrayed electrode

Silver enhancement and electrochemical impedance measurement

In a typical experiment, 10 μL of silver-enhancer solution was dropped onto the modified-electrode and the duration of the silver enhancement was set to 15 min. The electrode was then rinsed with water to remove the silver-enhancement solution.

The impedance spectra were recorded within the frequency range of 0.1 Hz–100 kHz. The number of frequencies recorded per frequency decade is 12. The amplitude of the applied sine wave potential in each case was 5 mV, while the direct current (dc) potential was limited at the formal potential of the redox pair $Fe(CN)_6^{3-/4-}$ (0.23 V vs. Ag/AgCl). The electrolyte solution was 5 mM $Fe(CN)_6^{3-/4-}$ (1:1) + 0.1 M KCl in 20 mM PBS (pH 7.6).

Fabrication of the arrayed ITO electrode and detection of antibody mixture

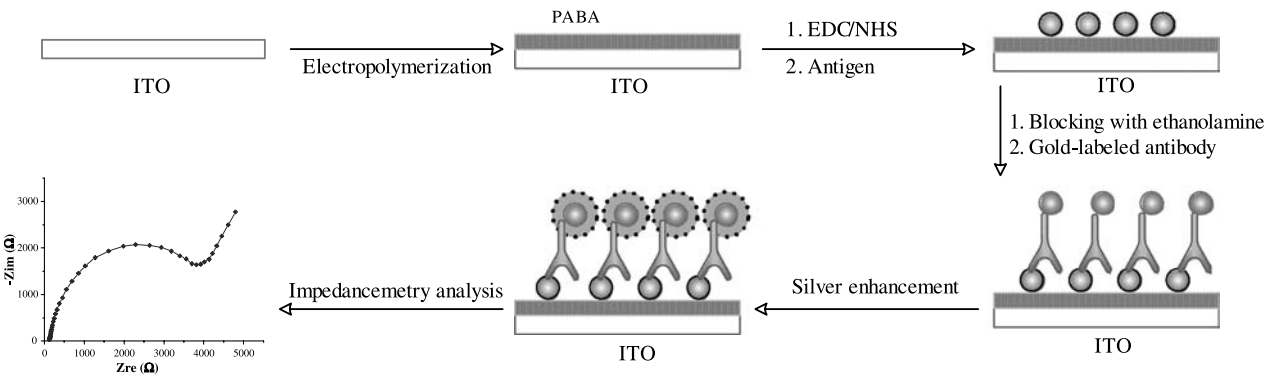
Scheme 1 shows the ITO arrayed electrode which contains four circular working electrodes (A, B, C, and D). For the detection of antibody mixture, mIgG was immobilized onto the two electrodes (A and C), while hIgG was immobilized onto the other two electrodes (B and D) using the above method. Then, 10 μL of antibody mixture containing gold-labeled goat anti-hIgG and goat anti-mIgG were dropped onto the whole ITO array surface and incubated at 37 °C for 2 h. Silver enhancement procedure and electrochemical impedance measurement were same as described above.

Results and discussion

Although most of immunological detection methods are based on the enzyme-label approach, metal-label methods such as gold nanoparticle tag have successfully been employed in electrochemical sensing methods including assaying nucleic acids [35] as well as proteins [36]. Recently, Yu and co-workers [37] reported a highly sensitive electrochemical impedance immunosensor for the detection of Human IgG with signal amplification based on Au-colloid labeled antibody complex. To further increase sensitivity, impedance detection based on silver enhancement was developed in this experiment and the schematic diagram of the stepwise self-assembly procedure of the immunosensor is shown in Scheme 2. Briefly, it consisted of five steps: (a) modification of ITO arrayed electrodes with electroconducting polymer (PABA) through electropolymerization; (b) immobilization of antigen on the PABA-modified electrodes; (c) blocking for nonspecific adsorption and interaction with gold-labeled antibody; (d) catalytic precipitation of silver onto the gold nanoparticles label using the silver enhancer solution; (e) electrochemical impedance measurement of the immune complex after silver enhancement.

Cyclic voltammetric studies of PABA-modified ITO arrayed electrodes

Figure 1 shows a typical cyclic voltammogram (CV) obtained during the electropolymerization process of PABA on the ITO arrayed electrodes. The shape of



Scheme 2. Schematic illustration of gold-labeled antibody recognition and signal amplification with silver enhancement

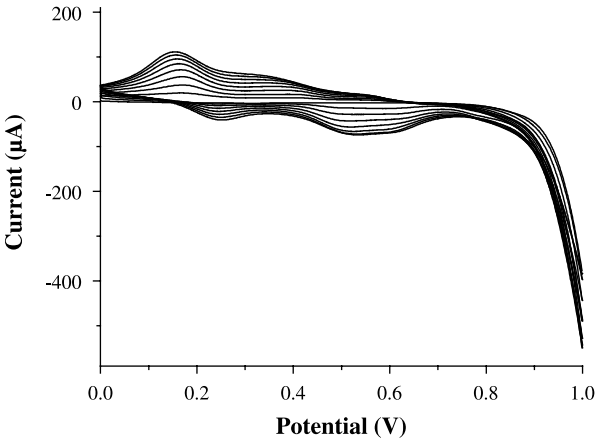


Fig. 1. The CV scan of the polymer film formed by electropolymerization of 0.05 M ABA in 1 M H_2SO_4 between 0 and + 1.0 V at a scan rate of 40 mV sec^{-1}

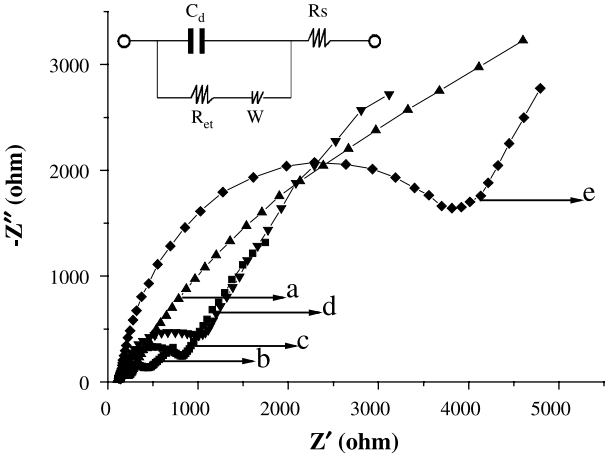


Fig. 2. Electrochemical impedance spectra of (*a*) bare ITO arrayed electrode, (*b*) PABA-modified ITO electrode, (*c*) after immobilized with Human IgG, (*d*) binding to gold-labeled goat-anti-normal human IgG, and (*e*) signal amplification with silver enhancement; at 0.15 V over a frequency range between 1 Hz and 100 kHz. The sinusoidal potential magnitude is + 5 mV in 5.0 mM $[Fe(CN)_6]^{4-}/^{3-}$ + 0.1 M KCl in 20 mM PBS (pH 7.6)

this CV curve is consistent with previously reported ones [40–42]. About 8 cycles were found to be enough to get the films with desired thickness. It can be seen that a sharp anodic peak at 0.155 V increases with electrochemical process and a shoulder occurs at 0.350 V, characteristic of the polymer oxidation. The electrical conductivity of the electroactive films is high enough to allow water discharge and, subsequently polymetric degradation takes place, giving a minimum current density at 0.750 V. On the negative sweep, two obvious cathode peaks can be seen at 0.250 V and 0.536 V which may be attributed to the reduction of the oxidized and electroactive PABA left on the electrode. All the electroconducting polymers used in this work have almost identical CV curves, indicating a good reproducibility of the PABA-modified electrode.

Electrochemical impedance characterization of the immunosensor

Compared with other electrochemical methods, the impedance technique has the advantage that the system is investigated under stationary conditions as opposed to the wide potential window used in CVs. The impedance technique can provide more detailed information about the interfacial properties of surface-modified electrode. In this experiment, electrochemical impedance spectroscopy (EIS) was used to monitor the fabricating process of the immunosensor, and the results are presented in Fig. 2. The impedance spectrum includes a semicircle portion at higher frequencies corresponding to the electron transfer limiting process and a linear part at the low frequencies resulting from the diffusion limiting step of the electrochemical process. The diameter of the semicircle exhibits the charge-transfer resistance of the layer, which shows its changed behavior for the redox couple. Thus, the semicircle diameter of EIS, which corresponds to the electrotransfer resistance (Rct), can be used to describe the interface properties of the electrode and its increasing value exactly characterizes the immobilization for each step.

It can be seen that the bare ITO arrayed electrode exhibits an almost straight line which implied the characteristic of a diffusion limiting step of the electrochemical process (Fig. 2a). A very small increase in Rct after coating of electroconducting polymer (PABA) indicates the good conductivity of the PABA-modified electrode (Fig. 2b). In the latter step, the immobilization of proteins generates the insulating protein layer on the assembled surface, which significantly enlarges the diameter of the semicircle, implying a higher electron-transfer resistance (Fig. 2c). After blocking with ethanolamine and reacting specially with gold-labeled antibody, the value of the Rct further increases, indicating the formation of immune complex (Fig. 2d). In the last step, silver enhancement is used to amplify the signal of the immunosensor, significantly enlarging the diameter of the semicircle in the impedance spectrum (Fig. 2e) and implying a high electron transfer resistance. As

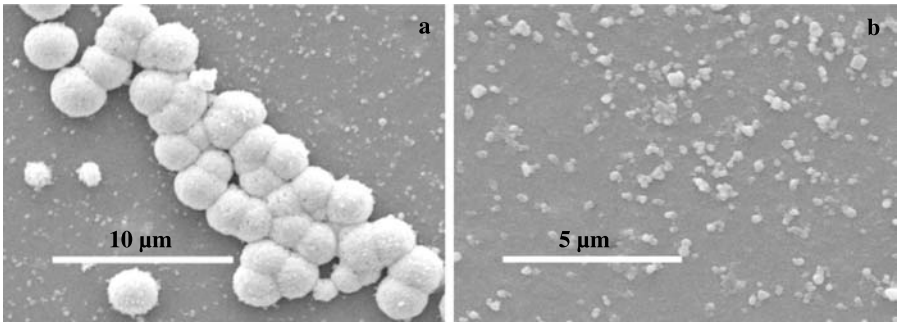


Fig. 3. The SEM images of different modified electrodes after silver enhancement: hIgG/PABA/ITO modified electrode after incubated with (a) and without (b) gold-labeled goat anti-hIgG. The gold-labeled antibody was 200 ng mL^{-1} and the silver enhancement time was 15 min

the silver ions in the silver-enhancer solution can be catalytically reduced exclusively on the gold colloids, resulting in thick silver layers on the surface of the modified electrode.

To further characterize the property of the ITO surface, SEM was also applied to the proposed silver enhancement process. As shown by the images in Fig. 3, the small gold nanoparticles were enlarged into large nanocomposites with gold core and silver shell, which suggested that the specific growth occurs after silver enhancement in our experiments. It is perceptible that when silver enhancement time reached 15 min, the size of silver nanoparticles in Fig. 3a was approximately 2, 3 μm and the volume was approximately 80 times larger than silver nanoparticles formed without gold nanoparticles core (Fig. 3b). It can be found that the silver layers onto the conductive electrode surface are dependent on the gold nanopartcles and this could greatly affect the charge transfer between the probe and the electrode. These phenomena were in accord with the results from electrochemical measurement above.

Optimization of immunosensor conditions

The capability of protein immobilization on the ITO arrayed electrodes was affected by the quality of the polymer film, such as the film thickness. Figure 4 shows the dependent relationship of the ratio of signal to background on the film thickness, which was controlled by the number of the electropolymerization cycles. The ratio increased rapidly with the electropolymerization cycles between 0 and 8, then decreased, and hence 8 cycles of electropolymerization was employed in the subsequent experiment.

Our results also indicate that the detection sensitivity is critically related to the silver enhancement reaction time. As shown in Fig. 5, the impedance values of target antibody together with the background were a

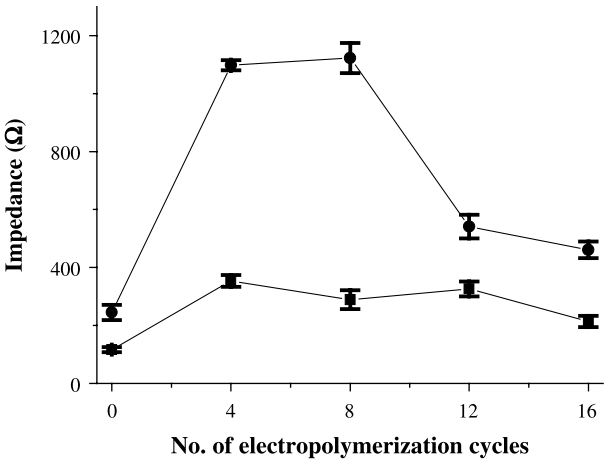


Fig. 4. The influence of the number of electropolymerization cycles on the impedance values of the PABA-modified ITO electrodes before (■) and after immobilized with hIgG (●)

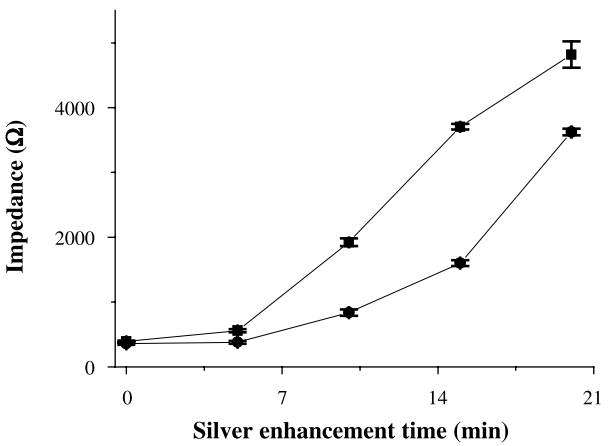


Fig. 5. The influence of the silver-enhancement time on the impedance values of the Human IgG/PABA/ITO modified electrodes after incubated with gold-labeled goat anti-hIgG (●) and gold-labeled goat anti-mIgG (■). The gold-labeled antibody was 200 ng mL^{-1}

function of the silver-enhancement time. It can be seen that little silver is deposited onto the gold nanoparticles within the first 5 min. With further increasing

silver-enhancement time, the impedance values of target antibody increase significantly and the detection sensitivity can be amplified by the extension of time. However, when the time is more than about 20 min, the ratio of signal to background approaches to saturation since a higher background can be formed. Considering the largest ratio of signal to background, a silver-enhancement time of 15 min was selected for all of the experiments.

Analytical performance

The fabricated immunosensor was utilized to assay gold-labeled goat anti-hIgG of various concentration and Fig. 6 shows the relationship between the charge transfer resistance change and the logarithm of concentrations of gold-labeled goat anti-hIgG for the ITO arrayed immunosensor. Under the optimized conditions, the charge transfer resistance (Rct) showed a linear correlation to gold labeled goat anti-hIgG concentration in the range of 0.01–10.0 $\mu g\,mL^{-1}$ with a regression equation: Rct = 1825.7 Log C ($ng\,mL^{-1}$) − 883.1 (R = 0.977). The detection limit was estimated to be 6.1 $ng\,mL^{-1}$ at 3σ. The sensitivity of this immunosensor was comparable to those for the immunosensors based on gold nanoparticles modified glassy carbon electrode (GCE) [43] and the immunosensors using enzyme labels as the amplifier [44].

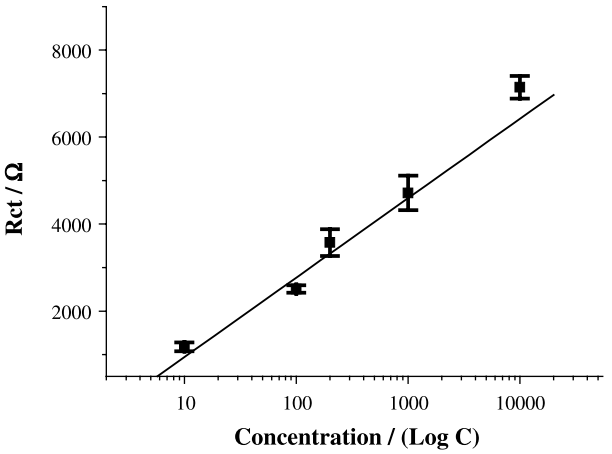


Fig. 6. The relationship between the electron transfer resistance change (Rct) and the logarithm of concentrations of gold-labeled goat anti-hIgG antibody for the ITO arrayed immunosensor

The ITO arrayed immunosensor was further developed to analyze multiple antibody-antigen interactions. In this experiment, two kinds of antigens, hIgG and mIgG, were selectively immobilized on the addressed arrayed electrodes through the immobilization frame. In order to investigate the specificity of the ITO arrayed immunosensor, the sample of 200 $ng\,mL^{-1}$ gold-labeled goat anti-hIgG was incubated with the arrayed electrode. Figure 7a shows the analytical results from the ITO arrayed electrodes. Compared to the mIgG control modified electrodes (A and C), the hIgG-modified electrodes (B and D) show higher impedance values after the incubation with 200 $ng\,mL^{-1}$ gold-labeled goat anti-hIgG based on silver amplification. The results suggested that the binding of hIgG on the immunosensor resulted in more antibody-colloidal gold conjugate bound to the electrode surface, leading to high impedance values after silver enhancement (curve B and D). While the immunosensor was incubated with mIgG, little colloidal gold was captured to the electrode surface, only small impedance values were observed (curve A and C), showing the non-specific adsorption was rather low. Without much non-specific adsorption, this immunosensor seems to have good selectivity. Moreover, when an antibody mixture (100 $ng\,mL^{-1}$ gold labeled

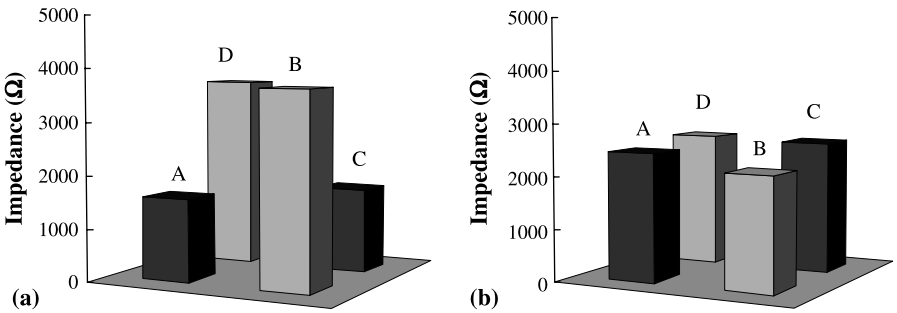


Fig. 7. The impedance values of the antigen-modified ITO arrayed electrodes after interaction with 200 $ng\,mL^{-1}$ gold-labeled goat anti-hIgG (a) and antibody mixture (b) The ITO arrayed electrodes were immobilized with mIgG (area A and C) and hIgG (area B and D). Antibody mixture contain 200 $ng\,mL^{-1}$ gold-labeled goat anti-hIgG and 200 $ng\,mL^{-1}$ gold-labeled goat anti-mIgG

goat anti-hIgG and 100 ng mL^{-1} gold labeled goat anti-mIgG) was incubated with the antigen array, the impedance values of the array were nearly the same (Fig. 7b). These results showed that (i) gold-labeled goat anti-hIgG and gold-labeled goat anti-mIgG could be selectively bound to hIgG and mIgG with no cross-talk phenomenon when antibody mixture was incubated with the arrayed electrodes and (ii) the electrochemical array based on the impedance method is promising for sensitively detecting antibody mixture in one sample.

Conclusion

In this paper, a new silver-enhanced colloidal gold electrochemical impedimetric detection based on the ITO arrayed electrodes offers great promise for the multiplexed immunoassay. To demonstrate the facility of this analytical approach, hIgG and mIgG were immobilized as capture proteins on the ITO arrayed electrodes by the utilization of electroconducting polymer (PABA) and then the mixture of the antibodies labeled with colloidal gold was reacted with the array. The signal amplification of the silver precipitation combining the inherent high sensitivity of impedimetric analysis was proven to provide ultrasensitive detection of multiple antibodies. The present analytical protocol shows an interesting potential for assaying real samples through an immunological competitive format. Thus, arrayed immunosensor based on ITO material has a great prospect in many fields such as clinical, environmental, and biodefense analysis since the quantitative analysis of multiple analytes is desired.

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