

## An impedance biosensor array for label-free detection of multiple antigen-antibody reactions

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## 1. ABSTRACT

An electrochemical impedance biosensor array with protein-modified electrodes was designed and fabricated in this report. To demonstrate its feasibility of the detection of multiple antigen-antibody binding reactions based on a label-free approach, human IgG (hIgG), rat IgG (rIgG), human globin and bovine serum albumin were immobilized, respectively, on the gold electrodes and then the resultant array was incubated with goat anti-hIgG, goat anti-rIgG, anti-human globin antibody and the mixture of three antibodies, respectively. The results indicated that the electron transfer resistance of the electrodes was significantly changed due to formation of the antigen-antibody conjugated layer. In addition, experimental conditions such as the protein concentration for the immobilization and screen were studied and optimized. Furthermore, the surface of various protein-modified electrodes was imaged with atomic force microscopy and the height distribution of protein particles was obtained with the Particle Analysis Software. The relative results were fully in accordance with the ones from the electrochemical impedance spectroscopy.

## 2. INTRODUCTION

High-throughput proteome analysis has been one of the important research fields since the drafting of the human genome. Compared to gene microarrays, protein microarrays can provide more direct information on interaction of protein-protein and DNA-protein (1-5). Parallel, high-throughput monitoring of interactions between biomolecules is playing a significant role in the characterization of various protein functions. Regular protein array technologies require label either analyses or the secondary antibody capable of binding on the analytes by using fluorescent compounds or radioisotope as the labeling markers, and this often causes some problems. First, chemical-labeling proteins could change their surface characteristics so that their natural activity would be impaired, especially for the small molecular proteins or peptides with few epitopes. Second, varied labeling efficiency for different proteins would cause the difficulty in accurately quantitative detection. Third, the labeling procedure is time consuming and labor intensive. As an alternative method, label-free detection method has its inherent merits including simple operation procedures and

avoidance of the disturbance from either conjugated labels or management for radioactive materials (6, 7). A typical label-free technique is Surface Plasmon Resonance (SPR) imaging technology, which is a promising tool to monitor biomolecular interactions of protein-protein, protein-DNA and DNA hybridization in real-time based on protein array or DNA array platforms (8-12). In addition, other new technologies such as atomic force microscopy (AFM), imaging ellipsometry, nanoscale interferometry and quartz crystal microbalance have also been developed to detect and screen the interaction of protein-protein (6, 7, 13, 14). However, expensive and complex equipments have to be required for these methods.

Electrochemical biosensor has many advantages including high-sensitivity, small dimension of the equipment and its easy integration (miniaturization) as well as inexpensive price. Though electrochemical measurement has commonly been coupled with immunological techniques such as ELISA and competition method, label procedures or labeled molecules have to be involved due to protein's electro-inactivity. As a consequence, most of electrochemical approaches such as amperometry and potentiometry have to be dependent on enzyme or other labels. These strategies could not only lead to an indirect analytical protocol, but also bring some difficult for development of a parallel assay with biosensors due to the crosstalk of the enzymatic products. In those cases, the products induced by the catalysis of enzymes could diffuse to the proximate electrodes and lead to the pseudopositive results. On the other hand, conductivity, capacitance and impedance measurements were reported to have the capability of direct monitoring of the change occurrence on the electrode surfaces (15). Compared to the former two methods, electrochemical impedance spectroscopy (EIS) is a more effective method to probe the interfacial properties of the modified electrode through measuring the change of electron transfer resistance at the electrode surface, which is caused by the adsorption and desorption of chemical or biological molecules. This label-free technique has been reported to assay biological targets such as proteins (16-18), DNA (19-21), aptamer(22), bacteria (23,24) and cells (25). To our knowledge, however, such an using biosensor arrays.

In this report, we explored the possibility of coupling impedance spectroscopy with a novel array protein-modified electrode. A gold array chip consisted of four protein-modified electrodes was designed and fabricated on the quartz substrate through vapor deposition technique. The antigen-antibody reaction as a model of protein-protein interaction was employed to evaluate the array biosensing method, in which multiple antibody-antigen interactions were carried out simultaneously and then measured by impedance spectroscopy. In addition, a series of experimental parameters such as protein concentrations for immobilization and sample screen were studied and optimized. In order to evaluate the electrochemical impedance measurements, AFM technology and Particle Analysis Software were also employed to obtain the height distribution of the protein

complex particles on the protein-modified gold electrode surface.

## 3. MATERIALS AND METHODS

### 3.1. Materials and solutions

2-Mercaptoethylamine (2-MEA) and Bovine serum albumin (BSA) were purchased from Sigma (St, Louis, MO); Glutaraldehyde was obtained from Beijing Chemical Reagents Co. (Beijing, China). Human IgG (hIgG) and Rat IgG (rIgG) were purchased from Bang Ding Biotech Co. (Beijing, China). Goat anti-human IgG and Goat anti-rat IgG were obtained from Sino-America Bioengineering Co. (Beijing, China). Human globin and anti-human globin antibody were kindly provided by South Medical University (Guangzhou, China). Other chemicals were of analytical grades and obtained from common commercial suppliers. The deionized water used in all experiments was produced by a Millipore Milli-Q water purification system.

### 3.2. Apparatus

All electrochemical impedance measurements were performed on a CHI 660 electrochemical workstation (CHI instrumental Inc., Austin, USA). A two-electrode system consisted of gold array working electrodes and a standard Ag/AgCl electrode served both as counter and reference electrode. The impedance data were fitted to an electrical equivalent circuit using the Zplot/Zview software (Scribner Associates Inc). All AFM experiments were performed using a SFM9500J3 Shimadzu, Japan and all images were treated with the Particle Analysis Software, Shimadzu, Japan.

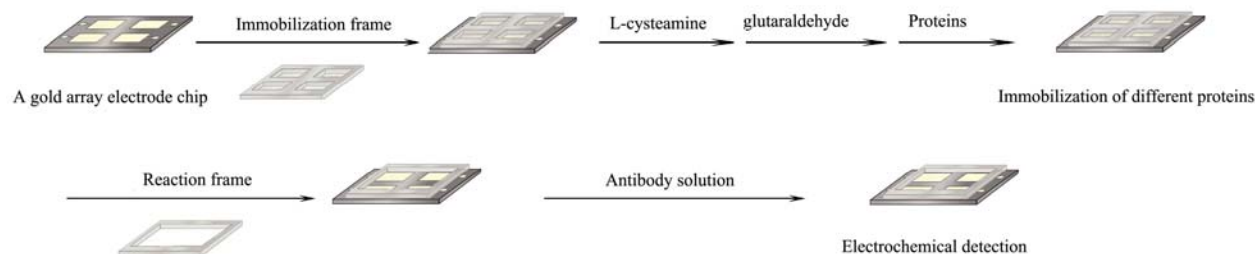
The electrochemical chip was prepared through using vapor deposition on the quartz substrate and the dimension of each single gold working electrode was 2mm×2mm (22). Prior to the modification, the chip was immersed in a piranha solution ( $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ : 3/1) for 30 min and then cleaned thoroughly with deionized water and ethanol, subsequently blown dry with high -purity nitrogen.

### 3.3. Experimental procedure

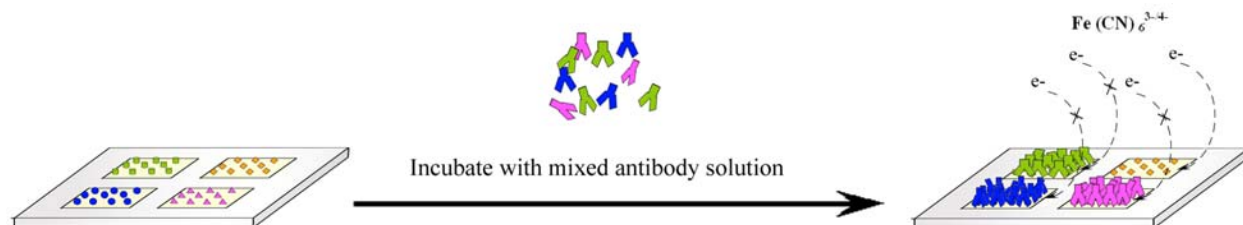
#### 3.3.1. Electrochemical measurements

The procedure of immobilization and analysis was illustrated in Figure 1. For immobilization of the proteins on the single gold electrodes, a frame containing the microwells was aligned onto the chip such that each well could exactly be matched the single electrodes. A 10mg/ml of 2-MEA aliquot was injected into each of the microwells within the immobilization frame and incubated for 4 hours. After the formation of a self-assemble monolayer (SAM) on the gold electrode surface, the chip was rinsed with deionized water twice and dried with nitrogen. Then, the modified chip was further incubated with glutaraldehyde (5%) for 1 hour, followed to rinse with deionized water. Finally, the four kinds of proteins (BSA, hIgG, rIgG, human globin) were delivered into the resultant microwells and incubated in the humid chamber for overnight.

## Label-free detection of multiple antigen-antibody reactions



**Figure 1.** Illustration of the immobilization and the analytical protocol.



**Figure 2.** Schematic illustration of the electron transfer blocking process on the gold electrode array surface.

After the protein bonding, the immobilization frame was removed and the chip was rinsed three times with a phosphate-buffer saline (PBS, 137mM NaCl, 3mM KCl, 10mM phosphate, pH 7.4), 5 minutes each time, following with water. Another frame for the incubation and detection procedure was attached on the protein-modified array. In this case, each sample would be reacted with four protein-modified electrodes simultaneously. A 50  $\mu\text{L}$  protein sample was induced into the analytical pool and incubated for 1h with the specific protein-modified electrodes, and then rinsed with PBS and dried with nitrogen.

Impedance measurements were carried out on CHI 660 electrochemical workstation using a two-electrode system. Prior to the detection, a 50  $\mu\text{L}$  of 5.0 mmol/L  $[\text{Fe}(\text{CN})_6]^{4-/3-}$  in 10 mmol/L PBS was pipetted into the analytical pool, and an Ag/AgCl electrode was positioned into the pool to form a circuit. The investigated frequency range was from 0.1Hz to 10 kHz at the formal potential of 250mV, using alternate voltage of 5mV. All electrochemical measurements were performed at 25°C.

### 3.3.2. Atomic force microscopy imaging

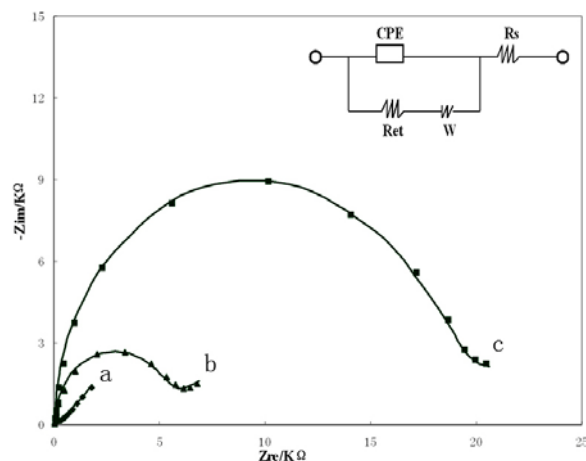
Preparation of the protein array was the same as the above description. Before performing AFM, the chip was rinsed thoroughly with PBS and deionized water to remove nonspecific adsorbed antibodies. All AFM experiments were performed using the tapping mode and all AFM images were further analyzed using the Particle Analysis Software.

## 4. RESULTS AND DISCUSSIONS

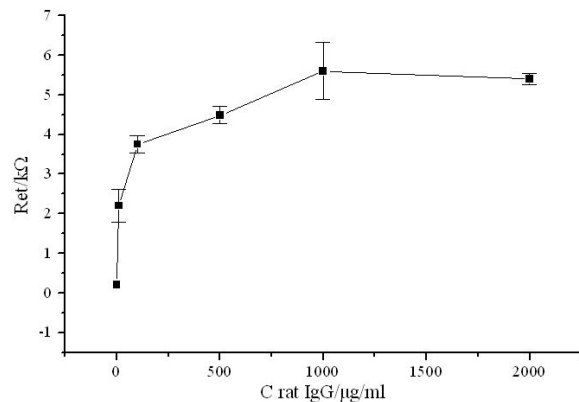
### 4.1. Electron transfer characteristics of the protein-modified gold array electrodes

The principle of label-free detection of the protein array using electrochemical impedance

spectroscopy is depicted in Figure 2. After incubating a sample containing the interested antibodies, the recognizing layer bonded the specific antigen would capture its specific antibody in the sample solution and form the antigen-antibody conjugated complex. Compared to the electrodes without the specific antigen, the conjugation layer on the specific antigen-modified electrode could not only increase the thickness of the modified layer of the electrode, but also lead to more compact of the surface. The resulting change would significantly reduce the electron transfer rate and the reduction extent would depend on concentration and affinity of the antibody. In order to acquire the data on the electron transfer rate, an AC impedance technique was employed and a Nyquist diagram of electrochemical impedance spectroscopy was displayed in figure 3. It can be seen that the Nyquist diagram includes a squeezed semicircle portion at higher frequencies, which corresponds to the electron transfer limited process, followed by a linear part characteristic of the lower frequency attributable to a diffusion limited process. The semicircle diameter equals  $R_{et}$  and the intercept of the semicircle with Zre axis at high frequencies is equal to  $R_s$ . In general, an impedance spectrum can be simulated using a Randles equivalent circuit which consists of electron transfer resistance ( $R_{et}$ ), redox solution resistance ( $R_s$ ), constant phase element (CPE) (When  $n = 1.0$ , it can be expressed as a double-layer capacitance) (24-26) and Warburg resistance ( $W$ ) (27-29). Ideally,  $W$  and  $R_s$  represent the properties of the bulk solution and the diffusion of the redox probe (marker). Thus, they are not influenced with the reaction occurrence at the electrode surface, whereas the CPE and  $R_{et}$  depend on the dielectric and insulating features at the electrode/electrolyte interface. Although the CPE and  $R_{et}$  are affected by the changes at the electrode surface, it has been found that the electron transfer resistance ( $R_{et}$ ) is the most direct and sensitive factor related to the electron transfer rate and responses on the interface (28), thus it often serves as the sensor signal in electrochemical



**Figure 3.** Nyquist diagram ( $Z_{im}$  vs  $Z_{re}$ ) for the faradaic impedance measurement of the gold electrode array in 10mmol/L PBS (pH 7.4) containing 5mmol/L  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  (a) without modification, (b) immobilized with rat IgG, (c) after the incubation with 10 $\mu\text{g}/\text{ml}$  goat anti-rIgG. The impedance spectra were recorded within a frequency range of 0.1Hz - 10 kHz. The amplitude of the alternate voltage was 5mV. Insert is the equivalent circuit, which represents each component at the electrode/electrolyte interface during the electrochemical reaction in the presence of the redox couple. Ret: electron transfer impedance;  $R_s$ : redox solution resistor; CPE: constant phase element; W: Warburg resistance.



**Figure 4.** The linear relationship between the electron transfer resistance and the concentrations of rIgG; the measurement conditions were same as those in figure 2.

impedance measurement. Figure 3 shows the impedance spectra of the electrodes (a) without modification, (b) immobilized with rat IgG and (c) incubation with goat anti-rat IgG after the immobilization. According to the Randles equivalent circuit (inset in figure 3), their Ret's are  $2.05 \times 10^2 \Omega$ ,  $5.3 \times 10^3 \Omega$  and  $1.89 \times 10^4 \Omega$ , respectively. The result demonstrates that rat IgG could be immobilized on the self-assemble monolayer of the electrode surface and further bound with the goat anti-rat IgG in the sample solution specifically. In addition, the electrode covered with an antigen-antibody conjugated layer could

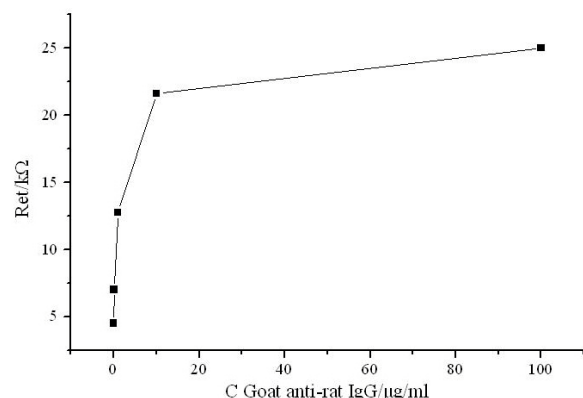
significantly increase the electron transfer resistance. As a result, it can be concluded from this experiment that the impedance measurement is an effective approach to the detection of antigen-antibody binding event without any label procedure.

#### 4.2. Optimization of experimental conditions

Because the goal of our work was to develop a method for direct screen detection of the interaction between antibodies-antigens, the investigation of background signals from the protein-modified electrodes would be of importance for read out of the effective signals. A series of experimental conditions such as the immobilization and detectable concentration of the protein were first compared. The relationship between the electron transfer resistance and the immobilization concentration of the protein is exhibited in Figure 4. It can be found that the electron transfer resistance increased continually with the rising concentration of rat IgG from 10 $\mu\text{g}/\text{ml}$  to 1mg/ml, indicating that more rat IgG was immobilized on the electrode with the rat IgG increment. When the rat IgG concentration arrives at 1mg/ml level, the electron transfer resistance reaches the maximum, suggesting that the protein covered all binding-sites for the modified electrode surface. Thus, 1mg/ml rat IgG was considered as the optimal immobilization concentration of the protein and used in the sequel experiments.

In order to explore the possibility of screening multiple antibody-antigen binding reactions using the protein modified gold array electrodes, the dependence of the background signals on the immobilization for various proteins was further investigated. In such an experiment without antigen-antibody conjugate, the electron transfer resistances for the four types of protein-modified electrodes using hIgG, rIgG, human globin and BSA were compared, and the electron transfer resistances were found to be  $4.8 \times 10^3 \Omega$ ,  $5.05 \times 10^3 \Omega$ ,  $5.61 \times 10^3 \Omega$  and  $4.85 \times 10^3 \Omega$ , respectively. The results indicate that there is a little difference of the impedance among the various protein-modified electrodes due to the discrepancy of the structure and size of the proteins. However, such discrepancy ( $\text{SD} < 20\%$ ) would not significantly affect the identification of the interaction of antigen-antibody affinity reactions.

The dependence of the impedance values on the concentration of protein samples was also investigated. In this experiment, a set of different concentrations of goat anti-rat IgG was used to incubate with the rIgG-modified electrodes to observe the impedance data. It can be seen from Figure 5 that the electron transfer resistance upsurges sharply with the rising concentration of goat anti-rat IgG until the concentration exceeding 10 $\mu\text{g}/\text{ml}$ . Then the electron transfer resistance growth slows down with the concentration continuous increment. Therefore, the protein in a low concentration range allows significant change in the impedance signal and this is easy to result in a relative large bias for the comparison of the interaction of multiple antigen-antibodies. Screening multiple antigen-antibody affinity reactions is the purpose of the report, thus the appropriate sample concentration is important to evaluate the binding event of the protein. As a result, the



**Figure 5.** The relationship between the electron transfer resistance and the concentrations of goat anti-rIgG; the measurement conditions were same as those in figure 2.

concentration of 10  $\mu\text{g/ml}$  was employed in the detection of protein-protein binding reactions in this experiment.

#### 4.3. Detection of multiple antigen-antibody binding reactions

A protein array was fabricated by immobilizing four different proteins of hIgG, rIgG, human globin and BSA on the array gold electrodes according to 3.3.1 section. In this case, each unit contains four types of biosensing electrodes. Fig.5 represents the screen results based on impedance spectroscopy, in which the modified proteins (antigens) were incubated with anti-hIgG (Fig.6a), anti-rIgG(Fig.6b), anti-human globin (Fig.6c) and their mixture samples (Fig. 6d), respectively. In these cases, BSA as a control was immobilized onto one of the four-electrode array. Therefore, compared to the other immobilized elements, its impedance value appears to be the lowest. On the other hand, when the antibody containing samples were incubated on the chip, the corresponding capture elements except BSA display a significant increment in impedance value and the increased values would be over twice than the background. This suggested that the impedance spectroscopy using protein modified electrode array could be used to assess antigen-antibody interactions.

It can also be observed from Figure 6(a) that there is some extent of cross-reactivity between the anti-hIgG and the human globin. This could be attributed to that the antibody belongs to the group of globins and has similarity to the human globin. In addition, goat anti-hIgG employed in this experiment was a polyclonal antibody and this allowed the possibility of binding with the human globins. As a result, anti-hIgG containing sample caused the impedance increment for the electrode modified with human globins. Conversely, the sample containing the anti-human globin antibody didn't lead to the impedance increase for the hIgG-modified electrode because the anti-human globin was monoclonal antibody (Figure 6c). When the protein array was incubated with the mixture of three kinds of antibodies, the electron transfer resistance for the hIgG, rIgG and human globin-modified electrodes all increased obviously (Figure 6d). However, the electron

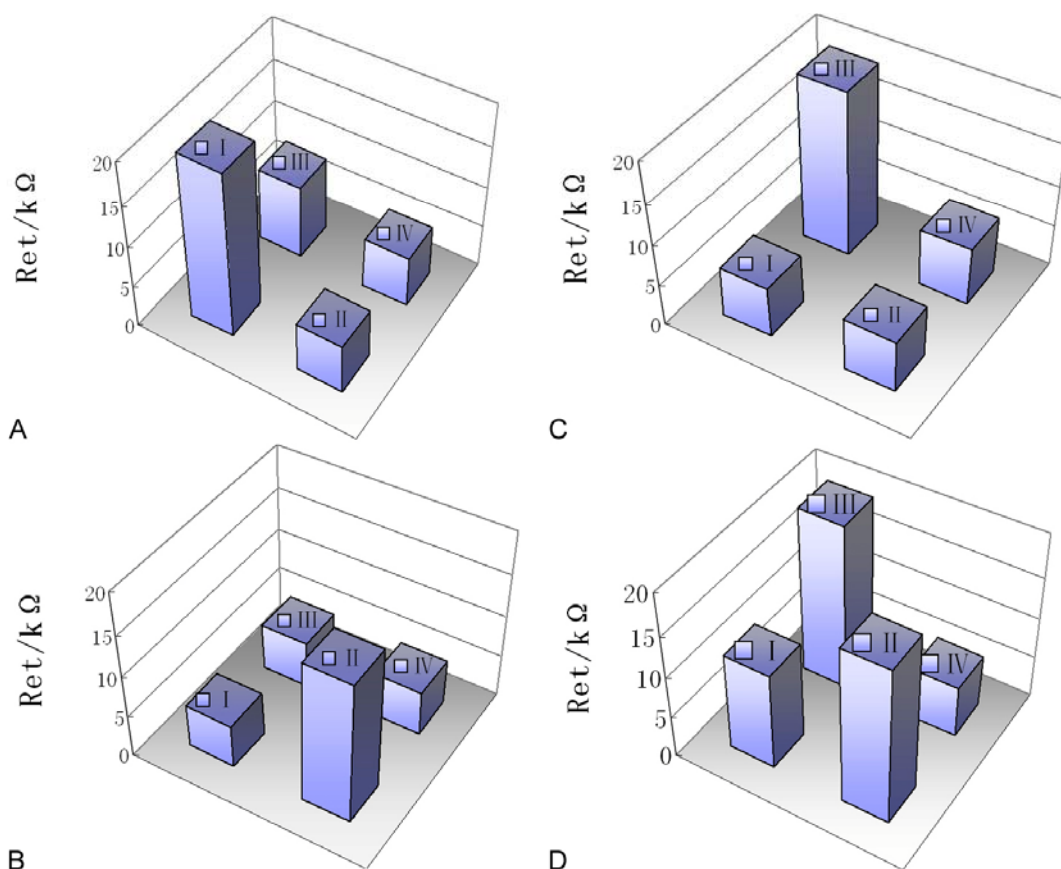
transfer resistance increment obtained from the human IgG-modified electrode was not the same significant as the other two electrodes, the reason might result from that the other two proteins in the mixture could hinder the binding between the goat anti-human IgG and the human IgG.

AFM imaging technology is a powerful tool to evaluate the bimolecular interactions at single molecular level, and has been applied to the detection of protein-DNA, receptor-substrate and antigen-antibody interactions (30-34). It was also reported to identify whether immobilized proteins bound with their antibodies and formed the relative antigen-antibody complexes (30, 32-33). In order to further verify the binding event on various proteins-modified electrodes and the results of the electrochemical impedance measurements, the protein array incubated with the mixture sample was imaged with AFM. Figure 7 shows the result of AFM measurement, and the height distribution of protein particles on the electrodes was further analyzed using Particle Analysis Software. As shown in the histogram of Figure 7, x-axis and y-axis represent the height and the number of protein particles, respectively. Because antibodies might associate with their antigens immobilized on the electrodes in different directions, the height for different the complexes displays a minor difference. It can be seen that the average height for the protein particles on the hIgG, rIgG, human globin-modified electrodes is around 13nm, and the average height of protein particles on the BSA-modified electrode is around 9nm. The difference in the height of particles on the electrodes between the ones binding the proteins and the control (BSA) is in accordance with the theoretical height of IgG molecules and the results indicate the existence of the specific complex on the electrodes (34). Compared to these electrodes, the BSA-modified electrode displaying weak hindrance for electron transfer rate did not show obvious nonspecific absorption (Figure 7d). In general, the result of AFM measurement further demonstrated the occurrence of multiple antigen-antibody binding reactions on the protein modified electrode array and the results agreed with the ones performed by the stated electrochemical impedance spectroscopy. Overall characteristics allow the electrochemical impedance technique using protein modified electrode array to be a simple and effective assay tool for researchers to efficiently investigate protein characterization / functions.

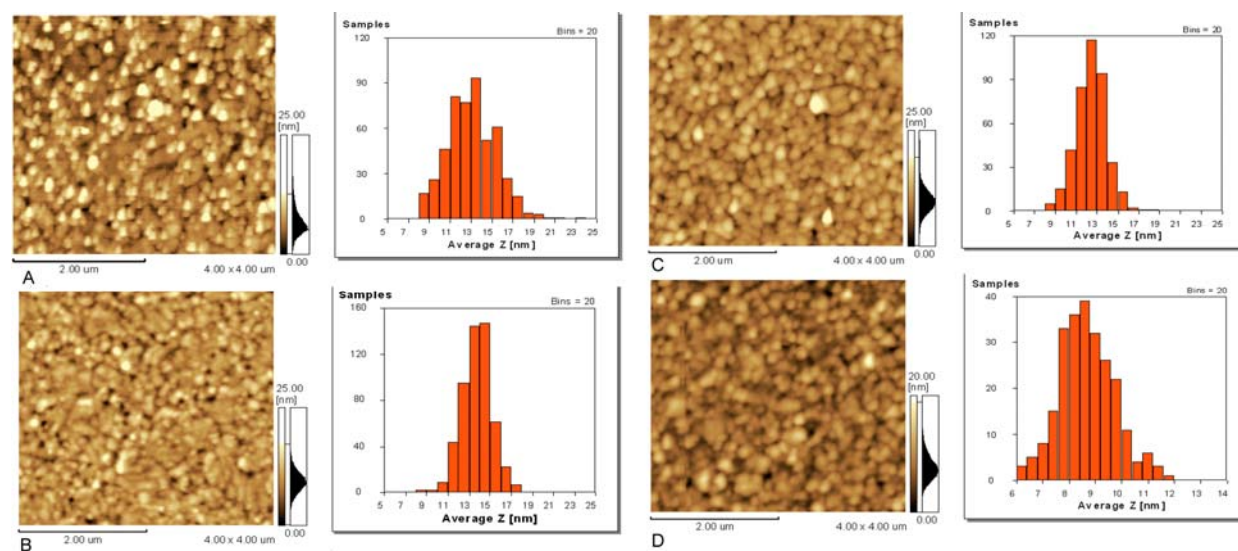
#### 5. CONCLUSIONS

In this paper, we demonstrated that the electrochemical impedance biosensor array is an effective tool for assaying multiple antigen-antibody binding reactions based on label-free screening strategy. The electrochemical chip with gold electrode array was fabricated and various proteins could be immobilized on the array through self-assembly plus protein immobilization techniques. Using electrochemical impedance measurement and the modified electrode array, multiple antigen-antibody binding reactions were assayed based on the optimal conditions. The binding events of the proteins on the electrodes were further proved by AFM technology. As a result, the present method can not only provide a novel





**Figure 6.** Screen results of multiple antigen-antibody binding reactions using electrochemical impedance spectroscopy. I, II, III and IV represent the immobilized proteins of hIgG, rIgG, human globin and BSA, respectively; (a), (b), (c), and (d) refer to post-incubation with goat anti-hIgG, goat anti-rIgG, anti-human globin antibody and the mixture of the three antibodies, respectively; each antibody concentration is 10 $\mu$ g/ml; the measurement conditions were same as those in figure 2.



**Figure 7.** AFM images (4 $\mu$ m $\times$ 4 $\mu$ m) of the gold electrode array after incubation with the mixture sample; (a),(b),(c) and (d) are the electrodes immobilized by hIgG, rIgG, human globin and BSA, respectively. The mixture consisted of goat anti-hIgG, goat anti-rIgG and anti-human globin antibodies; each antibody concentration is 10 $\mu$ g/ml; the right column represents the height distribution of the protein particles on the gold electrodes surface.

label-free electrochemical analytical approach in which multiple antigen-antibody interactions could be carried out in parallel, the sample and reagent consumption but also be saved to a great extent based on this integrating assay method. The method has a great promise for high-throughput screen for protein-protein and DNA-protein interactions. In addition, the research on developing more analytical functions such as dynamic and affinity analysis is in progress in our laboratory.

## 6. ACKNOWLEDGEMENT

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**Abbreviations:** hIgG: human IgG, rIgG, rat IgG, SPR: surface plasmon resonance; AFM: atomic force microscopy, EIS: electrochemical impedance spectroscopy, 2-MEA: 2-Mercaptoethylamine, BSA: Bovine serum albumin, SAM: self-assemble monolayer, PBS: phosphate-buffer saline, Ret: electron transfer resistance, Rs: redox solution resistance, CPE: constant phase element, W: Warburg resistance

**Key Words:** Impedance Spectroscopy, Electrochemical Biosensor Array, Label-Free Detection, Antigen-Antibody Interaction, Atomic Force Microscopy

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