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Simple and sensitive electrochemical impedimetric approach towards analysis of biophysical interaction

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ABSTRACT

Study of biophysical interactions have been carried out using specific combination of proteins such as human IgG (as antigen) and anti-human IgG (as complementary antibody; raised in rabbit). Bovine serum albumin (BSA) was used to block any nonspecific interaction between antigen and antibody as BSA has been reported to bind to several sites non-specifically. Optimization of BSA concentration was done in order to make the antigen–antibody interaction relatively more pronounced and specific. We have used gold electrode in order to provide a relatively inert platform for adsorption/immobilization of protein molecules. The interaction between the antigen and antibody caused an increase in the charge transfer resistance (parallel resistance in Randles cell model) for an indicator molecule (hexacyanoferrate) and this was monitored by impedance spectroscopy. Detection limit for the antigen was found to be about 50 ng/mL. The approach presented is general and versatile and can be used for any antigen–antibody pair without any significant modification.

1. Introduction

Several techniques like chemiluminescence [1,2], surface plasmon resonance [3,4], quartz crystal microbalance [5,6], absorption and fluorescence spectroscopy [7,8] and electrochemical techniques [9,10], have been developed and are widely used in immunoassay. Among all these techniques electrochemical immunosensor have been the subject of continued research and development in recent years [11–15]. Practically all immunoassay measurements depend on some form of ELISA (enzyme linked immuno-sorbent assay). Electrochemical immunosensors combine the high specificity of traditional immunochemical methods (e.g., ELISA) with the low detection limit and portability and convenience (point of care diagnostics) of a modern electrochemical system (e.g., glucose sensor). By using electrochemical methods, rapid, cost-effective and field-deployable diagnostic devices can be made.

Electrochemical immunosensors involves specific interaction between an antigen and their specific antibody. Electrochemical immunosensors can measure antigen–antibody binding in which potential [16], current [17] or resistance [13] is affected. In antigen–antibody binding analysis, changes in current or potential may be insignificant or very difficult to relate with particular antigen/antibody concentration in some cases where low concentrations of antigen (in case of antigen detection) or low concentrations of antibody (in case of antibody detection) are present. Antigen – antibody binding does not necessarily lead to a change in potential or current but can lead to a change in impedance, because the binding takes place close to the electrochemical double layer and affects the charge transfer rates. Measurements of impedance in antigen–antibody interaction analysis become more significant than measurements of change in current or potential. In electrochemical impedance spectroscopy technique (EIS), antigen–antibody binding is measured in terms of changes in the impedance developed close to the double layer [18]. The electrochemical impedance measured consists of series resistance (R_S), parallel resistance (R_P) (also called as charge transfer resistance), parallel capacitance (C_P) and Warburg impedance (W), assuming a simple Randles cell model. R_S is the characteristic of bulk electrolyte solution resistance (low significance), R_P and C_P are the double layer resistance and capacitance respectively and W is Warburg impedance taking part in diffusion controlled process. The antigen–antibody binding significantly affects R_P and C_P as they are taking place very close to the electrochemical double layer. The two other parameters (R_S and W) are not significantly affected by antigen–antibody binding [18–20]. The kinetically controlled

Abbreviation: BSA, bovine serum albumin; GE, gold electrode; PBS, phosphate buffer saline; EIS, electrochemical impedimetric spectroscopy; CV, cyclic voltammetry.

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process (charge transfer) is affected by antigen–antibody binding and is measured with the help of an indicator molecule (a redox couple is used in this study).

Electrochemical impedimetric spectroscopy (EIS) is a very sensitive and nondestructive electrochemical technique by which we can measure the interaction between antigen and its specific antibody without using labeled enzyme, as needed for ELISA [18,21–23] Compared with other sensors, impedimetric immunosensor is characterized by label-free, simple, time saving, requiring no special reagents, and can be used at lower potentials that avoid side reactions [18,24,25]. In impedance spectroscopy we apply a small sinusoidal alternating voltage (with any dc bias voltage if needed) and the resulting alternating current response is used to calculate the complex impedance (similar to resistance) at each of the applied frequencies. The amplitude of the potential and current values and the resulting phase difference between voltage and current dictates the overall impedance measured. Impedance results are commonly fitted to equivalent circuits of resistors and capacitors, such as the Randles circuit, which is often used to interpret simple electrochemical systems [18]. This equivalent circuit yields the Nyquist plot which provides some visual insight into the system dynamics. Once the interface is blocked due the antigen – antibody binding, the impedance increases. This can be accurately measured at some fixed bias potential.

By means of EIS, we study the process of immobilization of biocomponents (antigen and antibody) and characterize the electrochemical properties of bio-component/electrode interface. In EIS study, immobilization of bio-molecules on the electrode is the most important factor in fabricating high selective biosensor. Since the biological species (proteins in this case) are easily adsorbed onto the gold surface, we used gold electrode for the immobilization of bio-molecules in the present study.

In order to evaluate the electrochemical characteristic of modified electrode, potassium hexacyanoferrate ($[Fe(CN)_6]^{3-/4-}$) was selected as a probe or indicator molecule in cyclic voltammetry (CV) and EIS measurements. $[Fe(CN)_6]^{3-/4-}$ is a redox probe which can take part easily in the electron transfer kinetics close to the electrochemical double layer. When a bio-molecule is immobilized/adsorbed on the electrode surface, it retards the electron transfer rates between redox probe and electrochemical double layer, and hence, increases the charge transfer resistance for the redox probe to access the electrochemical double layer. This approach is very simple and versatile in principle and we tried to develop the detailed protocol and methodology. In principle, any suitable antigen–antibody pair can be analyzed by this method without any significant change of protocol.

2. Materials and methods

2.1. Apparatus

Electrochemical impedimetric spectroscopy (EIS) and cyclic voltammetry (CV) experiments were performed on a CHI660A electrochemical workstation. The experiments were carried out in a 10 mL conventional cell with gold electrode (GE) as a working electrode, an Ag/AgCl electrode as a reference electrode and a platinum wire as a counter electrode. The results have been plotted using Sigmaplot.

2.2. Reagents and chemicals

All chemicals used were of analytical grade and were purchased locally. Human IgG and anti-rabbit human IgG were purchased from Millipore (India). BSA was purchased from Sigma Chemicals (USA). Gold electrodes and polishing kit (containing alumina slurries of different size and polishing pad) were purchased from CH Instruments Inc. All Experiments were performed in a solution containing a mixture of 5 mM $K_4[Fe(CN)_6]$ and 5 mM $K_3[Fe(CN)_6]$. Phosphate buffer saline (PBS) containing 20 mM phosphate buffers with 150 mM NaCl (pH 7.4) was used in all experiments.

Purified human IgG was used as antigen and anti-rabbit human IgG was used as complementary antibody. Stock solution of human IgG (antigen) was diluted in PBS buffer of pH 7.4 as and when required. All dilutions were made freshly before use. It has been reported that pH 7.4 was optimal for the antibody immobilization [26].

2.3. Preparation of immunosensor

The bare gold electrode (GE) (2 mm diameter; CH Instruments) surface was polished repeatedly with 0.3 μm and 0.05 μm alumina slurries using micro-cloth polishing pad and was followed by rinsing with deionized water. Sonication in deionized water and ethanol respectively was done for 5 min followed by electrochemical cleaning. After drying the GE at room temperature, 10 μL of anti-rabbit human IgG (antibody; 50 μg/mL in PBS buffer) was deposited on the electrode surface and allowed to be immobilized on the surface of GE. The electrode was then left at 37 °C for 1 h, for immobilization to take place. The great advantage of physical adsorption is that no reagents are required for the immobilization and is satisfactory for single use. Only weak interactions are involved between the support and the bio-molecule due to van der Waals forces, dipole–dipole interactions, hydrogen bonding, or the formation of electron transition complexes. To remove the unbound/weakly bound antibody, the electrode was washed with PBS buffer and the non-specific binding sites on the antibody were blocked with optimized concentration of buffered BSA solution (100 μg/mL) for 30 min.

The BSA/antibody/GE electrode was allowed to interact with different concentrations of antigen in PBS solution for 15 min. Antigen/BSA/antibody/GE was washed with PBS buffer and is ready for impedimetric and cyclic voltammetry analysis.

2.4. Impedance & CV measurement

All electrochemical measurements were carried out in a conventional cell using a three electrode setup containing gold (bare/modified) electrode as working electrode, Ag/AgCl as reference electrode and a platinum wire as counter electrode. Experiments were carried out on CHI660A electrochemical workstation controlled by a personal computer. The data files produced by the electrochemical workstation were used in Sigmaplot to produce the final graph.

EIS and CV experiments were performed in 5 mM solution of $[Fe(CN)_6]^{-3/-4}$ at different stages of the preparation of the modified GE. Impedance spectra were recorded in the frequency range of 0.1 Hz–10 kHz. The amplitude of applied sine wave potential was 10 mV [27], while the direct current (dc) bias potential was limited at the open circuit potential measured just before the application of sine wave potential. CV experiments were performed in a potential range of −200 to 600 mV with a scan rate of 50 mV/s.

The electrochemical impedance spectra were analyzed using the ZSimpWin software (http://zsimpwin.software.informer.com/3.2/).

3. Results and discussion

3.1. Optimization of antibody concentration

Optimization of antibody concentration was carried out in order to maximize the output signal. In optimization process, various

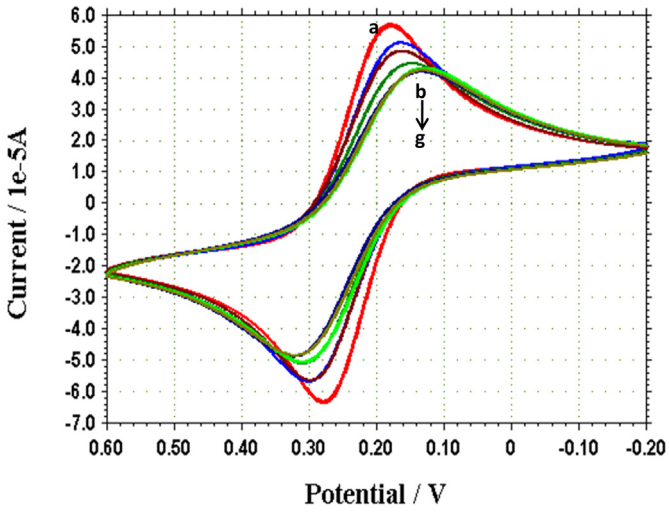

Fig. 1. Optimization of antibody concentration (anti-rabbit human IgG) in the range of 5–250 μg/mL. Curve (a) represent for bare gold electrode whereas curves (b–g) represent concentration range of antibody from 5 to 250 μg/mL.

concentrations of antibody (anti-rabbit human IgG) from 5 μg/mL to 250 μg/mL were tested using cyclic voltammetry (CV). Fig. 1 shows the various CVs of the GE modified with various concentrations (5, 10, 25, 50,100 and 250 μg/mL) of antibody in presence of 5 mM redox couple ($[Fe(CN)_6]^{-3/-4}$) as indicator. CV of the bare gold electrode showed a pair of well defined redox peak (curve (a) in Fig. 1), demonstrating rapid and reversible electron transfer. In Fig. 1, it can be seen that as the concentration of antibody on the GE surface increases from 5 μg/mL to 50 μg/mL, current steadily decreases (effective area of the electrode gets reduced).

The peak currents and positions are given in Table 1. It is seen that with increasing antibody concentration, the peak current decreases and the peak separation increases (reversibility of the charge transfer process decreases). This suggest that immobilization of antibody on the GE surface enhance the barriers in the transfer kinetics at electrode/electrolyte interface. Further increase in the concentration of antibody beyond 50 μg/mL causes no more significant decrease in the current. Therefore 50 μg/mL concentration of antibody was selected for further study.

3.2. Optimization of bovine serum albumin (BSA) concentration

BSA has been used to minimize the nonspecific interaction between antigen and antibody[18]. The use of BSA further increases the charge transfer resistance. Thus, the interaction of antibody with higher concentration of antigen can be easily distinguished (due to larger change in impedance) but the interaction of antibody with very low concentration of antigen became very difficult to quantitate. Therefore, in order to make the antigen–antibody interaction more visible, optimization of BSA was carried out in this study (CV figures used to optimize the concentration of BSA are not shown).

100 μg/mL concentration of BSA was used for optimization in this study. At this concentration of BSA, the difference in resistance between bare gold electrode and BSA modified gold electrode was found to decrease significantly (Fig. 2).

Table 1
The value of cathodic peak current (I_C), anodic peak current (I_A) and peak separation (E_C ~ E_A) corresponding to each concentration of antibody used in the CV study of optimization of antibody concentration.

Antibody concentration in μg/mL	Cathodic peak current in μA	Anodic peak current in μA	Peak separation in mV
0	65.04	70.05	97
5	56.29	62.68	137
10	52.77	60.08	143
25	46.29	53.46	161
50	39.02	49.59	191
100	41.60	51.94	187
250	40.88	49.65	189

3.3. Impedimetric detection of antigen–antibody interaction

EIS has been used to characterize and quantify the antigen–antibody binding on gold electrode. Diameter of the semicircle in the Nyquist plots of EIS represents the electron transfer resistance (R_P). Fig. 3 shows the EIS in Nyquist plots during the step by step modification of GE. It can be seen from Fig. 3, EIS of the bare gold electrode had a very small semicircle domain (curve a), indicating a very low R_P, and hence, implying low resistance for electron transfer of the redox probe dissolved in the electrolyte solution. After modification of gold electrode with optimized concentration of antibody (curve b), semicircle diameter of EIS in Nyquist plot became larger than that of bare gold electrode. When further modification of GE with BSA (curve c), followed by antigen (curves d–k) were carried out, the semicircle diameter of EIS in Nyquist plot increased steadily. This is the result of the increase in charge transfer resistance for redox probe to access the gold electrode (GE) surface, owing to the adsorption of bio-molecules which retards the electron transfer kinetics between the redox probe and the electrode surface [8]. The curves (d–k) in Fig. 3 represent the Nyquist plots for the antigen concentration from 50 ng/mL to 50 μg/mL. With the antigen concentration of 50 ng/mL and less, no more significant decrement in semicircle diameter in Nyquist plot was observed. Thus, the minimum concentration (or detection limit) of antigen in antigen–antibody interaction detected by EIS in this study was 50 ng/mL.

3.4. Quantitative analysis of EIS results

Quantitative analysis of EIS results for antigen–antibody interaction was done according to the equivalent circuit $R_S(C_P||(R_PW))$. Quantitative analysis of EIS results made this EIS study of bio-

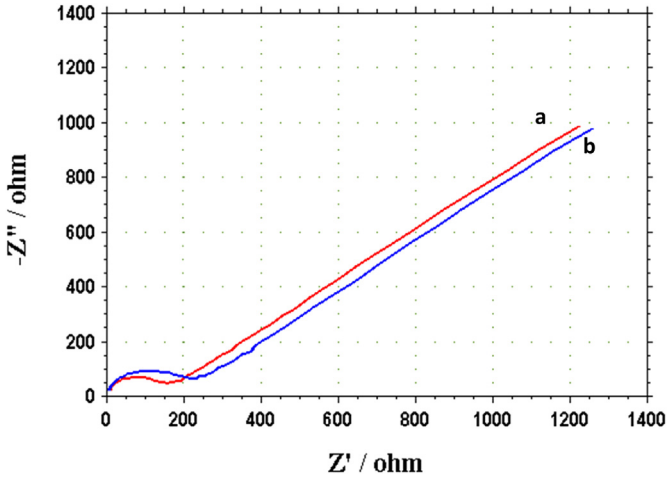

Fig. 2. Comparison of EIS results. Curve (a) represents bare gold electrode whereas curve (b) represents BSA(100 μg/mL) modified gold electrode. Z′ and Z″ represent the real and imaginary components of the impedance respectively.

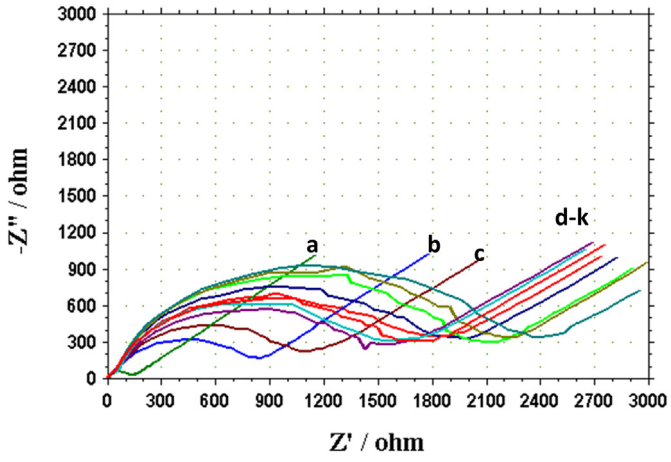

Fig. 3. EIS result for biomolecular interactions. Curve (a) represents bare gold electrode, curve (b) represent gold electrode modified with antibody (50 μg/mL), curve (c) represents BSA (100 μg/mL)/antibody (50 μg/mL)/gold electrode, curve (d–k) represent antigen concentration from 50 ng/mL to 50 μg/mL.

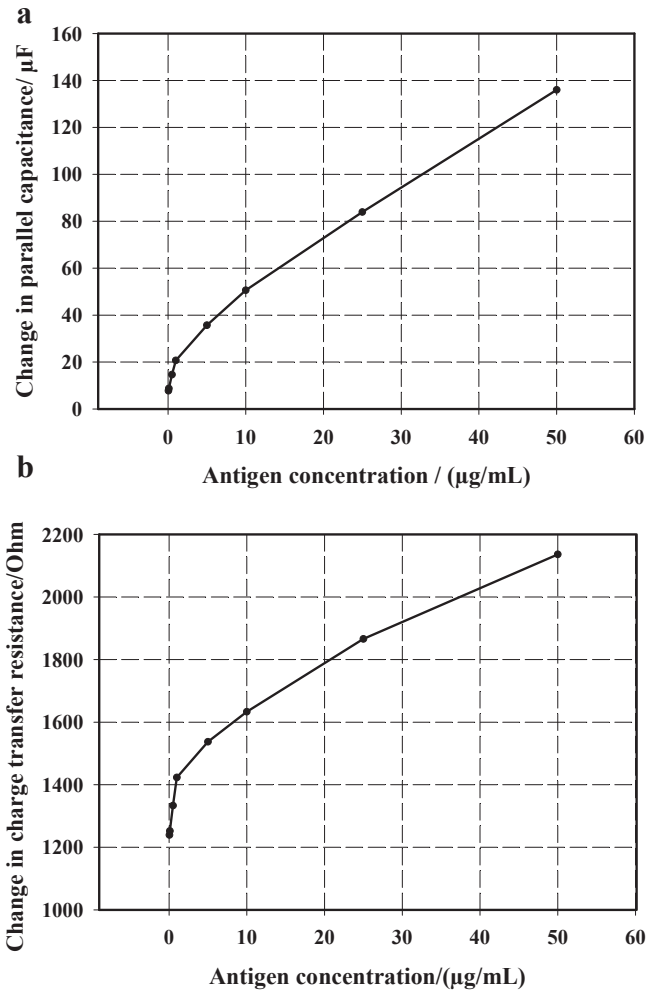

Fig. 4. (a). Calibration plot between change in parallel capacitance (ΔC_P) and antigen concentration. (b). Calibration plot between change in parallel resistance (ΔR_P) and antigen concentration.

molecular interactions more authentic, unique and reliable. In quantitative analysis, C_P (parallel capacitance) and R_P (parallel resistance or charge transfer resistance) values were calculated from EIS results of antigen–antibody interaction. Two calibration plots were obtained; one plot between ΔC_P (C_P without antigen subtracted from C_P with antigen) vs. antigen (human IgG) concentration (Fig. 4a) and another plot between ΔR_P (R_P without antigen subtracted from R_P with antigen) vs antigen (human IgG) concentration (Fig. 4b). The detailed values of the parameters are given in Table 2. The R_S represents the series resistance in the bulk solution and hence, not significant in the present EIS study. As expected the Warburg impedance is quite small and represents the resistance in the diffusion layer and does not depend on the antigen–antibody binding process.

Table 2
Changes in Cp and Rp values with respect to the application of particular concentration of the antigen on BSA/antibody/GE. The values of R_S and Warburg impedance corresponding to each applied concentration of antigen also shown in table.

Antigen concentration (μg/mL)	R_S (Ohm)	ΔCp (Farad)	ΔRp (Ohm)	W (Ohm)
0.05	4.731	7.726E-6	1239	0.0005811
0.1	5.501	8.704E-6	1252	0.0006117
0.5	5.344	1.463E-5	1433	0.0005921
1	5.668	2.072E-5	1423	0.0006002
5	4.808	3.5692E-5	1537	0.0006103
10	5.497	5.058E-5	1633	0.0006051
25	5.858	8.394E-5	1866	0.0005984
50	4.901	1.361E-4	2136	0.0006137

Thus, in this study, we have tried for a unique approach for analysis of bio-molecular interaction. In this study, optimization of BSA concentration also plays a unique role in order to analyze the bio-molecular interaction in very low concentration. Besides this, study of parallel capacitance and charge transfer resistance for antigen–antibody interaction further increases the uniqueness of the approach carried out in this EIS study. Study of parallel capacitance and charge transfer resistance also increases the authenticity and reliability of this study.

Competing financial interests

The authors declare no competing financial interests.

Conflict of interest

The authors declare no conflict of interest.

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