Differential Impedance Spectroscopy for Monitoring Protein Immobilization and Antibody–Antigen Reactions

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This work describes the theoretical and experimental approaches for monitoring the interfacial biomolecular reaction between immobilized antibody and the antigen binding partner using novel differential impedance spectroscopy. The prerequisite of any biosensor is the immobilization of macromolecules onto the surface of a transducer. It is clear that the function of most macromolecules changes from what is observed in solution once immobilization has occurred. In the worst case, molecules entirely lose their binding activity almost immediately after immobilization. Certain conditions (e.g., denaturation, interfacial effects based on ionic strength, surface charge, dielectric constants, etc.) at interfaces are responsible for alterations of binding activity; it is not clear whether a combination of such processes is understood. However, these processes in combination must be reliably modeled in order to predict the outcome for most macromolecules. This work presents the theoretical and practical means for elucidating the surface reactivity of biomolecular reagents using ion displacement model with antibody–antigen (Ab–Ag) reaction as the test case. The Ab–Ag reaction was directly monitored using a dual-channeled, impedance analyzer capable of 1 measurement/s using covalent immobilization chemistry and polymer-modified electrodes in the absence of a redox probe. The evidence of Ab–Ag binding was revealed through the evolution of differential admittance. The surface loading obtained using the covalent immobilization chemistry was $9.0 \times 10^{15}$/cm$^2$, whereas with polymer-modified electrodes, the surface loading was $9.0 \times 10^{15}$/cm$^2$, representing a 10 times increase in surface reactivity. The proposed approach may be applicable to monitoring other surface interfacial reactions such as DNA–DNA interactions, DNA–protein interactions, and DNA–small molecule interactions.

In recent years, different conceptual approaches have been reported for the realization of bioaffinity sensors, which are capable of detecting the specific reaction between a receptor and its ligand. Electrochemical detection principles have been of only minor significance with respect to direct sensing. Yet, electrochemical impedance spectroscopy (EIS) represents one of the most powerful methods for the investigation of interfacial reaction mechanisms.1–4 This work presents the theoretical and practical means for elucidating the surface reactivity of biomolecular reagents using differential impedance spectroscopy (DIS). The potential of detecting surface recognition reactions via DIS especially with no redox probe is presented.

EIS provides a rapid approach for monitoring the dynamics of biomolecular interactions, and it can be used to predict important aspects of biosensors including surface reactivity, surface loading, binding constants, and rates of reaction. It can contribute to the interpretation of fundamental biochemical processes via exact mathematical and physical models that are based on plausible linear and macroscopic phenomena. Bioaffinity reactions can take place in solution or with one molecule of the binding pair attached to the surface of a solid biosensor surface. The affinity of a ligand (present in solution) to an array of many different immobilized receptors (e.g., biosensors) is a potentially promising analytical tool. In theory, the identity and amount of an unknown ligand can be distinguished by the binding constant of the antibody–antigen (Ab–Ag) interaction. These biosensors are capable of detecting a single analyte from a labeled, complementary binding partner such as those found in clinical, environmental, and biological agents of mass destruction. Numerous affinity biosensors that use EIS for proteins, DNA–DNA, antibody–antigen (Ab–Ag), or oligonucleotide–DNA interactions5–8 have been reported. In these biosensors, heterostructures (such as antigen...
antibody, cells, DNA, enzyme layers, and semiconductors/thin silica layer) are immobilized at the surface of a solid electrode. The binding of the reacting partner is then verified through the detection of either a shift in impedance, change in capacitance, or admittance at the bulk of the electrode interface. A key step of this sensing protocol is the immobilization of the bioaffinity reagents onto suitable substrates. The technique for attaching reagents to the substrates includes covalent attachment and bifunctional linkage to the adjacent layers of the polymers such as polycyramide. It is also possible to take advantage of the conductivity of conjugated polymers onto a solid electrode surface.

EIS has been extensively used to study cells that are arbitrarily shaped and to simulate the evolution of the suspension of initially synchronized budding yeast. In electronic cell—substrate impedance sensing, the impedance increases because the cells attached to the working electrode act as insulating particles to restrict the flow of current. We previously used EIS to study Ab—Ag interactions at conducting polymer-modified electrodes and other surfaces. Recently, we reported the supramolecular docking and immobilization of biotinylated dsDNA onto a self-assembled monolayer of avidin using EIS and QCM techniques. We found that, upon binding to the biotinylated dsDNA, the interfacial electron-transfer resistance of small organic particles could be monitored through changes taking place in the electron-transfer resistance at an unmodified electrode relative to the variable electron-transfer resistance introduced by the multilayered DNA. This interaction was monitored in situ, and a detection limit of low magnitude (nM) was achieved.

Despite the potential advantages of EIS to study biomolecular reactions, very few of such practical biosensing systems are currently available. Major fundamental issues still exist due to possible ambiguities associated with data interpretation and the complication of analyses based on the equivalent circuits. In addition, compared to other biosensors that are based on optical or microgravimetric principles, the sensitivity of direct impedimetric biosensor is relatively low. This may be attributed to the slow rate of data collection when the existing device is used and the nonlinear and nonstationary property at the interface. Unlike simple chemical systems, ideal electrical circuits may be inadequate to describe the surface distribution, the microscopic properties, and the electronic behavior of the biomolecular interactions. Consequently, the application of EIS has been severely limited to corrosion and interfacial processes at simple chemical interfaces. In this paper, we report the use of dielectric spectroscopy of a differential biosensor for the detection of two different Ag-modified surfaces in phosphate buffer solutions (PBS) and in the samples, the DIS signal between the real and the imaginary components was amplified by a factor of 1000, thus resulting in a dramatic change in sensitivity. We emphasize that the reference electrode employed is similar to the active sensing electrode except that this electrode has been modified with either an inactivated polymer or another Ab (e.g., not specific to the target Ag). Since the impedances of both electrodes are similar, the differential signal (carrying the information on specific binding) can be amplified at a factor much larger than the signal across the individual sensor. This is the basis of the enhanced sensitivity of DIS detection.

**PRINCIPLE OF DIFFERENTIAL IMPEDANCE SPECTROSCOPY**

EIS provides an electronic measurement method in which the complex spectra of the electronic behaviors of a sample are collected and analyzed using proper software routines. This method is advantageous because it is essentially a steady-state technique in which the relaxation phenomena could be assessed over several orders of magnitude. To minimize nonspecific effects and issues associated with the integrity of biomolecular orientation, we present the use of an ion displacement model based on impedance spectroscopy. We are interested in monitoring the dynamics of Ab—Ag interactions using impedance measurements of the modified electrodes in a differential arrangement. The differential setup offers a significant advantage in comparison with a single-channel system due to the possibility of high amplification. In this case, the signal containing the desired information is amplified by eliminating the influence of background contribution resulting from temperature, electrolyte composition and nonspecific coupling while compensating for the impedance effects of electrode/bulk. From a basic electrical scheme, with impedance elements within the interface in parallel, the equivalent impedance of a probe, Z\text{probe} is given by

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(b) Taylor, R. F. Protein immobilization fundamentals and applications; Marcel Dekker Inc.: New York, 1991.
\[ Z_{\text{probe}}(t) = Z_{\text{electrode}} + Z_{\text{interface}}(t) + Z_{\text{bulk}}; \\
Z_{\text{probe}}(0) = Z_{\text{electrode}} + Z_{\text{i}}/N + Z_{\text{bulk}} \quad (1) \]

where \( N \) is the number of possible binding sites. Assuming no nonspecific coupling, \( N \) corresponds to the number of Ab present at the electrode surface. The term \( Z_{\text{electrode}} \) in eq 1 represents the impedance of the sensor that is not affected by the Ag–Ab coupling. It consists of the impedance of the metal, the polymeric layer, and the entrapped Ab not affected by Ag coupling. Let us assume that a reference (blank) and an active sensor (where the Ab–Ag reaction takes place) both exhibit similar values of the \( Z_{\text{electrode}} \). In the differential arrangement, the \( Z_{\text{interface}} \) gives the major contribution, i.e., the impedance of the electrode–bulk interface that “hosts” the immobilized Ab reagent. To minimize the effects of electrode polarization on \( Z_{\text{interface}} \), frequencies above 10 kHz were considered. We therefore assumed that the voltage drop across the interface is the same all over the surface of the working electrode.

During the process of Ab–Ag interaction, we consider that the impedance, \( Z_{\text{i}} \) of any newly bound antigen, replaces the impedance, \( Z_{\text{0}} \) of the electrolyte that has previously occupied that locus. The basic assumption of our theory emphasized the existence of a relationship (the same whenever the same immunosensor is used) between the admittance (impedance) due to Ag–Ab coupling and the admittance (impedance) of the bulk that has been withdrawn from the interface. In the particular case of a one-to-one binding scheme, for \( N_{1} \) the number of active Ab present at the surface of the working electrode, and \( n(t) \) denoting the number of Ag bound at time \( t \), the impedance, \( Z_{\text{interface}} \), and admittance, \( Y_{\text{interface}} \) of the interface, are given by

\[
\frac{1}{Z_{\text{interface}}(t)} = \frac{n(t) + N_{1} - n(t)}{Z_{0}} \quad \leftrightarrow \\
Y_{\text{interface}}(t) = n(t) Y_{1} + (N_{1} - n(t)) Y_{0} \\
dY_{\text{interface}}(t) = dn(Y_{1} - Y_{0}) \quad (2) 
\]

Therefore, the larger the real and imaginary components of the difference of \( Y_{1} - Y_{0} \) become, the better the sensitivity of this method. Assuming the following time dependence

\[
dn/dt = K(N_{1} - n(t)) \Rightarrow n(t) = N_{1}(1 - e^{-Kt})
\]

if

\[
K = \frac{N_{V}}{V} = \frac{n(t)}{1 - N_{1}/N_{0} e^{rac{-N_{V} - N_{0}}{Vt}}}
\]

where \( N_{V}/V \) is the volume concentration of antigens in solution.

One can derive the dynamics of the process of binding in relation to the concentrations of Ag/Ab in the experimental setup. When a differential measurement of the working electrode (WE) versus a blank electrode (BK) is carried out, the differential admittance can be obtained from

\[
\Delta Y = \frac{Y_{\text{BK}}(t)}{Y_{\text{WE}}(t)} - 1 = \frac{Z_{\text{WE}}(t) - Z_{\text{BK}}(t)}{Z_{\text{BK}}(t)} = \\
Y_{0} \frac{1}{Y_{1}} \frac{1}{n/N_{1} + (1 - n/N_{1})Y_{0}} - 1 \quad (4)
\]

\[
Z_{0}/Y_{1} = \frac{Y_{0}}{1/R_{0} + IwC_{0}} \\
Z_{1}/Y_{1} = \frac{1/R_{1} + IwC_{1}}{1/R_{1} + IwC_{1}} \\
Y_{0} = 1R_{0} + w^{2}C_{0}C_{1} + Iw(C_{0}R_{1} + C_{1}R_{0}) \left(1/R_{1}^{2} + (wC_{1})^{2}\right) \quad (5)
\]

Consequently, the progress of the Ab–Ag reaction can be revealed directly by rearranging eq 4 to give

\[
n(t) = \frac{\Delta Y(t)}{(1 + \Delta Y)(1 - Y_{0}/Y_{1})} \quad (6)
\]

where \( \Delta Y \) is the measured data and \( Y_{0} \) and \( Y_{1} \) are the admittances of the bulk electrolyte and the related, newly bound Ag, respectively. The ratio \( Y_{1}/Y_{0} \) depends on the chosen Ab pair, Ag, and the type of immobilization procedure employed. The immobilization approach will provide different orientation for the active sites on the Ab. Therefore, one should expect different values of \( Y_{1}/Y_{0} \) for Ag with different molecular weights or orientation (e.g., IgG and cyanazine). The left-hand side of eq 6 represents the ratio of bound Ag versus surface-bound Ab, while the right-hand side comprises the measured admittance values (\( \Delta Y \)) using EIS. The sign of \( \Delta Y \) is related to the ratio \( Y_{1}/Y_{0} \) whether below or above 1. Besides \( \Delta Y(t) \), this method is able to provide a direct way to validate the theory by checking the imaginary component of the EIS experiment according to

\[
\text{Im} \left[ \frac{\Delta Y}{(1 + \Delta Y)(1 - Y_{0}/Y_{1})} \right] = 0 \quad (7)
\]

Moreover, eq 6 assumes that there are no linear relationships with concentration. However, it is possible to equate the nonlinear relationships in eq 6 with linear terms by expanding

\[
n(t) = \frac{\Delta Y(t)}{(1 - Y_{0}/Y_{1})} \quad (8)
\]

**EXPERIMENTAL SECTION**

Reagents and Stock Solutions. All reagents are analytical grade unless otherwise stated. Nanopure water with a resistivity of 17 M Ω-cm was used for the preparation of all aqueous solutions. Potassium phosphate (dibasic and monobasic) was from Fisher Scientific Co., and potassium ferrocyanide (K,Fe(CN)₆) was from ACE Scientific Supply Co. Cystamine dihydrochloride (2,2-diaminodiethyl disulfide), 1-ethyl-3-(3-dimethylaminopropyl)carboimidate (EDC), (3-aminopropyltriethoxysilane (APTS), glutaraldehyde (GA), 25% solution in water), and bovine serum albumin (BSA) were from Sigma; and anti-cyanazine antibody solutions were made in phosphate-buffered saline containing 0.05% Tween 20 (PBST), pH 7.4. Pyrrole (98%) was obtained from Aldrich,
stored at 4 °C, and distilled before use. Immunoreagents were as follows: anti-sheep IgG (from rabbit) of 5 mg/mL total protein and sheep IgG with total protein amount of 20 mg/mL (both purified and concentrated), and human-IgE (Pasteur Institute, Bucharest, Romania). The immunoreagent stock solutions were divided into aliquots and stored in the freezer at −18 °C. The basic phosphate buffer saline solution (pH 7.4) used for the preparation of all other solutions contains 0.067 M KH₂PO₄ (Merck), 0.067 M Na₂HPO₄·12H₂O (Chimopar S.A), and 1.5 × 10⁻¹⁴ M NaCl (Chimopar S.A.).

**Antibody Production.** The design and preparation of analyte analogues and immunogens are essential steps in the development of low molecular weight immunosensors. Cyanazine hapten (MW 310) was a gift from Dr. James Fleeker of North Dakota State University. These were prepared from active esters of the carboxylic acid analogues of triazine hapten using N-hydroxysuccinimide. We coupled these triazine hapten to high molecular weight carriers, BSA and keyhole limpet hemocyanin (KLH), which served as the antigen. Using these haptons and protein-linked conjugates, polyclonal cyanazine antibodies (SB372) were raised in rabbits under contract with Cocalica Biologicals Inc. of Pennsylvania. The antibodies were purified using gel filtration and protein-A immunoaffinity columns and subsequently characterized using ELISA and nuclear magnetic resonance (NMR) techniques.

**Instrumentation.** Electropolymerization experiments were carried out using a galvanostat designed and produced by the International Center of Biodynamics (ICB) in Bucharest, Romania. The driving software of the galvanostat was also developed at ICB and was interfaced to the galvanostat through a personal computer. Galvanostatic electropolymerization experiments were conducted in a three-electrode cell, consisting of a platinum wire as working electrode (area, 0.1 cm²), saturated calomel electrodes as reference electrodes, and a platinum sheet as the auxiliary electrode. A dual-channeled impedance analyzer, which was also designed and produced at ICB, was used for the impedance assays. This analyzer provided the time series of the real and imaginary components of the measured impedances at a high accuracy (Δφ = 0.01°) and 0.5 mV in amplitude at low currents, ~5 µA, with options for averaging and rate of data collection at 1 measurement/s.

Comparative tests on passive elements were performed using Agilent 4294A impedance analyzer, Solartron 1260 impedance analyzer, and EG&G Lock-in amplifier model 5210, combined with an EG&G PAR 273 potentiostat/galvanostat equipped with M 398 software. A sinusoidal potential modulation of ±10 mV amplitude was superimposed on a dc potential. The amplitude and phase shift of the resulting current were recorded at each frequency from 65 kHz to 1 Hz. Fifteen points equally spaced on a logarithmic scale were acquired per decade increment in frequency (f).

**Sensir Preparation and Characterization. Immobilization of IgG Antibody.** Anti-sheep (IgG) was entrapped in a polypyrrole (PPy) membrane that was obtained by galvanostatic polymerization using platinum as support. The area of the electrodes was 0.1 cm² unless otherwise stated. The PPy-anti-sheep IgG films were prepared using a solution containing 0.5 M pyrrole and 100 ppm anti-sheep IgG in the PBS solution. The electropolymerization experiments were carried out using an electrochemical cell containing 0.5 M Ppy in a total volume of 8.5 mL of PBS (pH 7.4) purged with N₂ for 10 min before the addition of anti-sheep IgG. The applied current density was 0.3 mA/cm². During galvanostatic electropolymerization of PPy-anti-sheep IgG, the charge passed was 180.0 mC. The electrodes containing anti-sheep IgG entrapped in the PPy membrane were incubated in PBS—sheep IgG (100 ppm) solution overnight at 4 °C for 24 h.

**Immobilization of Anti-Cyanazine Antibody.** Anti-cyanazine antibody was immobilized onto solid electrodes (Au and Pt) in two ways: either by entrapment into a PPy conducting polymer or by connecting to the electrode via covalent attachment with APTS.

Electrochemical Immobilization via Polymer Entrapment. The solid electrodes were pretreated by boiling in 2 M KOH solution for 2 h. Immediately before any hapten or antibody immobilization, the electrode were thoroughly rinsed with water, soaked in concentrated nitric acid for 10 min, and rinsed again. Antibody entrapment was achieved using an electrochemical microassembly as previously described. A 1-mL aliquot of SB372 was diluted to 5 mL with PBS (pH 7.4), and using the platinum working electrode, a constant oxidation current of 3 mA/cm² was applied for 10 min. Then a SB372/PP film was obtained and was incubated in a SB372 solution (SB372/PP = 1:4) for 24 h. This preparation served as a working electrode designated as Pt/PPy/antibody.

Immobilization via Covalent Attachment. The antibody could be covalently attached to the electrode surface through the amide bond (by using APTS) as shown in Figure 1. A 5% APTS solution in aceton was prepared. A drop of this solution was applied onto a gold electrode and dried. This procedure was repeated twice. Then the electrode was incubated in the oven at 100 °C for 1.2 h. After cooling, the electrode was rinsed with acetone and then incubated in a 1:4 SB372 solution overnight. This preparation served as a working electrode designated as gold/APTS/antibody electrode.

**Determination of Surface Density of Active Binding Sites Using EIS.** The differential EIS was employed to estimate the surface reactivity after immobilization has occurred. Figure 2 shows the setup employed during EIS experiments. A sinuousoidal signal of small amplitude was applied to the working electrode and the current response measured and controlled potentiostatically. The impedance was calculated as the ratio of the system voltage phase (vector), U(ω)/I(ω), and the current phasor, I(ω), which are generated by the frequency response analyzer during the experiment, where ω = 2πf (with units in rad s⁻¹) and f is the excitation frequency (Hz) as shown in eq 9.

\[
Z(jω) = U(jω)/I(jω) = Z_{re}(ω) + jZ_{im}(ω)
\]
the antibody surface loading, (ii) to calibrate the biosensor, and (iii) to monitor the dynamics of the biomolecular reaction. As discussed in the theoretical section, we can quantitate the Ab surface density or loading by assuming that, during the process of Ab–Ag reaction, the impedance, $Z_1$, of any newly bound antigen replaces the impedance, $Z_0$, of the amount of electrolyte that previously occupied that locus. Meaningful determination of the surface density of active sites is reasonable only under the condition of saturation. Hence, electrolyte concentrations that provide a saturation condition could be determined by measuring the impedance of the solution (blank) prior to exposing the electrode to Ag solution and estimating the difference after exposure to the antigen.

Characterization and Monitoring of Biomolecular Reactions Using Differential Impedance. Cyclic voltammetry was performed on the polymer-modified electrodes using an EG&G

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**Figure 1.** Stepwise assembly of covalent immobilization of antibody on a solid Au electrode. The APTS was a 5% solution in acetone; antibody solution was made up in PBS buffer pH 7.4.

**Figure 2.** Schematic diagram of the experimental setup for differential impedance spectroscopy. The differential amplifier can be connected to the same voltage input or variable input at both its inverting and noninverting inputs to produce the amplified difference between two input voltages. The electrochemical cell consists of blank electrode (or noncoated probes) and the sensing electrode that has been modified with specific antibodies (or antigen). The two electrodes are connected to the same voltage source; the variable resistor is adjusted to the point at which the output voltage becomes zero. In this way, the noise and other background picked up in wires or other parts of the system can be reduced.
potentiostat/ galvanostat. The cyclic experiments were performed in a solution of PBS pH 7.4. The sweep rate was 100 mV/s for all CV experiments. The biochemical activity of the sensing electrode was assessed via enzyme-linked immunosorbent assay (ELISA) following a method developed by Bender et al.13 Wells of rigid polystyrene plates were coated (overnight at 4 °C) with 50 µL of a 50:50 mixture of a 1:500 dilution in carbonate buffer (pH 9.6) of monoclonal antibodies SB 372. Glass coverslips, 15 × 15 × 1 mm, were coated by immersion and incubated in 200 µL of a 1:1000 dilution of SB 372. The experiments were performed in triplicate: a 1:5 dilution in PBST and antigen preparation of cyanazine was serially diluted in 2-fold steps across the monoclonal antibody-positive rows and control (negative rows) of the ELISA plate. After incubation for 2 h at room temperature, the plate was washed with PBS buffer and distilled water, air-dried, further treated with secondary antibody (1:250, 50 µL/well, 2 h/37 °C), washed, and quantified with alkaline phosphatase-conjugated IgG and chromogenic substrates following manufacturers’ instructions. The plate was read in BioRad ELISA plate reader. From the ELISA experiments, the optical binding affinity of the immobilized antibody was determined by plotting the absorbance versus the concentration of coating antigen for various antibody concentrations (checkerboard titration).

Monitoring Ab–Ag Interactions. The response of immunosensors to the presence of the target analytes (sheep IgG and cyanazine–BSA) was investigated by differential impedance analysis. The impedance instrumentation provides the difference (of both real and imaginary parts of the impedance as described under the theoretical section) between a blank and a working electrode. Either or both electrodes in the active cell can contain PPy—anti-sheep IgG or cyanazine antibody membrane. All experiments were carried out at 10 kHz unless otherwise stated. The electrodes were immersed in PBS (pH 7.4) in 3.0-mL cell. The impedance response of the immunosensor is due to the Ab–Ag coupling.23,24,35,36 To establish the working range of the sensors to the presence of the target analytes (sheep IgG and cyanazine),0.9–1.2 mV was assessed via enzyme-linked immunosorbent assay (ELISA) experiments, the optical binding affinity of the immobilized reagent and to overcome the problems of sensor reproducibility.

Surface Density of Active Binding Sites Using EIS. It is clear that the function of many macromolecules changes from what is observed in solution once immobilization has occurred. In the worst case, molecules entirely lose their binding activity almost immediately after immobilization. While it is known that, the reproducibility of the calibration curve was determined within a limit of 10%.

RESULTS AND DISCUSSION

Immobilization Study. To demonstrate the proof of concept of the differential impedance approach, we first studied the immobilization chemistry employed. An ideal sensor must be characterized and optimized in all its different aspects to fulfill the demand for enhanced sensitivity as predicted in the theoretical section. This stipulates that the surface activity, and hence the molecular recognition, must be maintained once immobilization has occurred. The retention of the molecular recognition properties of the immobilized biomolecules requires that the integrity of orientation be maintained on the solid phase.37 Although biomolecules effectively bind to solid surfaces,38 they may be denatured in the process39 and thus lose their biospecific activity.40 In the case of antibodies, spatial orientation of antibodies onto the supports that might prohibit the formation of an Ab–Ag complex could result from normal immobilization schemes.41 The CV voltammograms of the PPy—antibody-modified electrodes were similar to those observed elsewhere,41 indicating the formation of conductive polymers. Figure 1 shows the covalent immobilization chemistry employed for the antibody-immobilized electrode. ELISA experiments performed for antibody-incorporated conducting electroactive polymers indicated that the optimal antigen binding concentrations were from the steepest slope in the linear region of the curve. The polymers prepared by electrochemical techniques were found to produce activities between 0 and 1. Any polymer with absorbance values outside this range was considered inactive for sensing purposes. This approach simple rule can generally be used to assess the activity of the immobilized reagent and to overcome the problems of sensor reproducibility.

Cross Reactivity and Reproducibility. The selectivity of this method has been proven by using different concentrations of other proteins, as well as measuring the EIS responses. Similar response impedance curves of antigen concentrations were obtained by adding IgG after an initial concentration of 10 ppm human IgE.

Table 1. Determination of Surface Density Using Differential Impedance Spectroscopy

<table>
<thead>
<tr>
<th>sensor</th>
<th>$Z_i / Z_1$</th>
<th>$N_i$ (molecules/cm²)</th>
<th>$n_i$ (molecules/cm²)</th>
<th>$\Delta Y$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt/PPy/antibody (cyanazine–BSA sensor)</td>
<td>0.89 – j0.035</td>
<td>9.0 × 10¹⁵</td>
<td>9.0 × 10¹⁵</td>
<td>0.12 + j0.044</td>
</tr>
<tr>
<td>Au/APT/antibody (cyanazine–BSA sensor)</td>
<td>0.90 + j0.023</td>
<td>9.0 × 10¹⁶</td>
<td>9.0 × 10¹⁶</td>
<td>0.11 – j0.028</td>
</tr>
<tr>
<td>anti-IgG-modified electrode</td>
<td>0.97 – j0.0084</td>
<td>4.8 × 10¹⁴</td>
<td>4.8 × 10¹⁴</td>
<td>0.03 + j0.009</td>
</tr>
</tbody>
</table>

* Polyferrrope entrainment immobilization, ** Covalent immobilization approach.

under certain conditions such as denaturation, interfacial effects based on ionic strength, surface charge, dielectric constants, etc., at interfaces are responsible for alterations of binding activity, it is not clear whether a combination of such processes is understood. However, it is clear that these processes in combination must be reliably modeled in order to predict the outcome for most macromolecules. Assuming a 1:1 Ab/Ag ratio at saturation, we estimated $N_1$, the number of active antibodies present at the working electrode and $n(t)$ the number of antigen (Ag) at time $t$, from the impedance data according to eq 6. The saturated surface coverage of the Ab was obtained using the one-to-one binding relation and the Ag concentration corresponding to the beginning of the plateau. Table 1 shows the surface density determined at the modified surfaces. The surface loading obtained using the covalent immobilization chemistry was $9.0 \times 10^{15} \text{ cm}^{-2}$, whereas with the polymer-entrapped electrodes, the surface loading was $9.0 \times 10^{15} \text{ cm}^{-2}$. This represents a 10 times increase in surface reactivity for the Au/ APTS/ antibody electrode. This is reasonable, since in this electrode, the antibody has been covalently linked to the Au surface and this approach may provide preferred orientation than the Pt/PPy/antibody electrode in which the antibody was only entrapped into the pores of the polypyrrole layer. The saturation value of $\Delta Y$ enables the determination of the ratio $Y_1/Y_0$ in calibration experiments as

$$Y_1/Y_0 = 1 - \frac{\Delta Y_{sat}}{1 + \Delta Y_{sat}}.$$ (10)

In most experiments, the saturation value lies between 3.5 and 4.0 ppm. For cyanazine sensors, comparable saturation was recorded at both the covalent-linked electrode versus the PPY-modified electrode.

**Monitoring the Biomolecular Reaction and Sensor Calibration.** The IgG and cyanazine electrodes were used in the detection of IgG and cyanazine–BSA analytes, respectively, using the fast dielectric spectroscopy. Theoretical consideration followed the description provided earlier. As predicted, the interaction of the antibody electrode with different antigen concentrations resulted in the formation of an Ab–Ag complex that produced measurable impedance changes. The dynamics of these reactions could be observed continuously from time dependencies of impedance changes of the ICB impedance analyzer (Figure 2). A Solartron impedance analyzer attached to a personal computer was used to follow the frequency changes as described in the Experimental Section. The extent of the interaction was found to depend on the surface loading (antibody concentration) and the time of exposure to the antigen solution. The binding amount at equilibrium was evaluated with respect to the impedance changes since the amount of antibody loading at the surface is known.

We measured the changes in impedance when electrolyte solutions were added instead of the complementary antigen pair. We determined the amount of Ag that bound and the electrolyte ions released based on the ion displacement model. The change in admittance could be directly measured ($\Delta Y$), the sign being related to the ratio $Y_1/Y_0$. We previously reported that the real part of the impedance decreased using human serum albumin sensors based on capacitance measurement. In the present work, the change in the real part of the impedance ($\Delta Z_{re}$) was found to be dependent on the antigen concentration. The concentration dependence of the $\Delta Z_{re}$ is shown in Figures 3–5. In Figure 3, the linear range for the antigen (IgG) was between 0.4 and 3.5 ppm, with a detection limit of 0.1 ppm. In Figure 4, the linear relation between $\Delta Z_{re}$ (Pt/PPy/ antibody) and antigen (cyanazine–BSA) concentration was obtained between 0.1 ppm and 2 ppm with a linear coefficient of 0.99. The detection limit was 0.01 ppm. For the measurements of 200 ppb solutions ($n = 6$), the relative standard deviation was 2.4% A similar relationship was found for the Au/ APTS/ antibody electrode. Figure 5 shows the response of the Au/ APTS/ antibody electrode when exposed to antigen solutions. The linear domain was between 1 ppb and 20 ppm with a linear coefficient of 0.97 and the detection limit of 0.1 ppb. For the measurements of 10 ppm solutions ($n = 5$), the relative standard deviation was 4.0%.

It was found that when the Pt/ ppy/antibody electrodes were exposed to the cyanazine–BSA antigen, the real part of the impedance decreased. It was assumed that the Ab/Ag reaction could facilitate the reduction process of the PPy polymer hence decreasing the impedance. After the antibodies were saturated by the antigens, any additional antigen added might become incorporated into the polypyrrole layer as counterions, resulting in further decrease in impedance. Since the incorporated antigens would be closer to the Pt surface than those at the polymer–bulk interface, the decrease of impedance would be faster; i.e., $\Delta Z_{re}$ would be expected to have a sudden increase, as shown in Figure 4 when the antigen concentration was above 2 ppm.

Typical DIS responses obtained for the PPy–anti-sheep IgG-modified electrode before and after exposure to different concentrations of antigen solutions are shown in Figure 3. The decrease in the DIS response of the antibody monolayer to the corresponding antigen solution for a fixed time produced a quantitative measure of the antigen concentration. The lowest detection limit achieved for the IgG sensor was 0.1 $\mu$g/mL, 0.01 ppm for the cyanazine sensor with a response time of a few minutes, and less than 2% cross-reactivity to atrazine, simazine, and other metabolites. The response of the anti-cyanazine antibody-modified electrodes to other potential interferences such as proteins or compounds with similar structures was tested (Table 2). The electrodes had no response for the following: 100 ppm BSA, 10 ppm prometon, 5 ppm tebuthylazine, and 5 ppm desethylatrazine. This observation was recorded for both the Pt/PPy/antibody and
Au/APTS/antibody electrodes; OVA: 20 ppm for Pt/PP/antibody and 2 ppm for Au/APTS/antibody electrode. From the data, it could be observed that the electrodes only responded to the hapten side of the conjugates (cyanazine).

**SUMMARY AND CONCLUSION**

Direct observation of biomolecular reactions by fast differential impedance spectroscopy is demonstrated using covalent immobilization chemistry and a series of polymer-modified electrodes without a redox probe. Ab–Ag binding is revealed by the evolution of the differential admittance. The biosensor achieves nanogram per milliliter sensitivity for IgG and cyanazine. The advantages of this method are that relatively high speed and high precision are attainable. We have emphasized the use of fast differential impedance spectroscopy for both the characterization of the surface density and monitoring the biomolecular reaction. This emphasis is justified by the need for fast quality control after immobilizing the bioreagent and enhanced sensitivity. The detection limits recorded were in the low-nanomolar range. To study the presence of proteins on the solid electrodes after immobilization, we utilized differential impedance spectroscopy and demonstrated in a qualitative way the immobilization of the antibody and antigen.

**Table 2. Summary of Cross-Reactivity Data**

<table>
<thead>
<tr>
<th>cross reactant</th>
<th>concentration, %</th>
<th>Pt/ PPy/ antibody</th>
<th>Au/ APTS/ antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td></td>
</tr>
<tr>
<td>prometron</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td></td>
</tr>
<tr>
<td>teubuthylazine</td>
<td>&lt;0.4</td>
<td>&lt;4</td>
<td></td>
</tr>
<tr>
<td>desethylatrazine</td>
<td>&lt;0.4</td>
<td>&lt;4</td>
<td></td>
</tr>
<tr>
<td>OVA</td>
<td>&lt;0.1</td>
<td>&lt;10</td>
<td></td>
</tr>
</tbody>
</table>

* Experimental conditions: The electrodes were fabricated as in Experimental Section. The cross-reactivity studies were conducted as reported for the sensing electrodes in PBS (pH 7.4).

Au/ APTS/antibody electrodes; OVA: 20 ppm for Pt/ PP/ antibody and 2 ppm for Au/ APTS/ antibody electrode. From the data, it could be observed that the electrodes only responded to the hapten side of the conjugates (cyanazine).

**Figure 4.** Response of Pt/PP/antibody electrode to cyanazine–BSA antigen in PBS (pH 7.4). In EIS, the frequency was kept around 10 kHz and the ac amplitude was 200 mV. The inset shows the linear calibration part of the data. Other conditions are as shown in Figure 3.

**Figure 5.** Response of Au/APTS/antibody electrode to cyanazine–BSA antigen. The inset shows the linear calibration part of the data. The conditions were as in Figure 4.
its subsequent affinity binding to the antigen. After calibration, an exact quantitative comparison between the different immobilization procedures is possible using DIS. For this purpose, we described an ion displacement model as a theoretical approach to explain the Ab–Ag binding. The results are consistent with the theoretical prediction presented. The proposed method combines the capabilities of EIS and bioaffinity reactions for accurate and rapid detection of the binding events on the surface of modified electrodes in a differential setup.

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