Monitoring of the avidin–biotinylated dextran interaction on Au- and Ti/TiO$_2$-electrode surfaces using a charge integrating device

Spiros D. Bolis$^a$, Panagiota C. Charalambous$^a$, Constantinos E. Efstathiou$^{b,*}$, Aikaterini G. Mantzila$^b$, Constantina A. Malamou$^b$, Mamas I. Prodromidis$^b$

$^a$ Department of Chemistry, University of Athens, University Campus, Athens 157 71, Greece
$^b$ Department of Chemistry, University of Ioannina, Ioannina 45 110, Greece

Received 19 January 2005; accepted 4 April 2005
Available online 17 May 2005

Abstract

In the present paper we report the use of a homemade electronic device (Multipulser), for monitoring interactions between biomolecules that may change the capacitance of an electrode. Multipulser can be used as a stand-alone, low-cost, yet effective alternative monitoring device instead of other well established commercial instruments. The operation of Multipulser is based on the integration of the electric charge used for the repetitive charging of the electrochemical cell capacitance after the application of a predetermined number of short-duration, low-amplitude voltage pulses (perturbation pulses). Multipulser was used to monitor the binding of biotinylated dextran on two different avidin modified electrode assemblies, one based on a thiol SAMs on gold and another based on Ti/TiO$_2$ semiconductor. Measurements conducted in parallel with a commercial frequency response analyzer gave similar reaction patterns. Pulse polarity dependent behavior was revealed in the case of the Ti/TiO$_2$-electrode assembly when bipolar potential perturbation modes were used with Multipulser.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Charge integration; Capacitance immunosensors; Impedance measurements; Monitoring of avidin–biotin interactions

1. Introduction

The development of methods targeting the direct monitoring of antibody–antigen interactions or biotin–avidin complexes formation, which is commonly used as an amplification strategy of aforementioned immunoreactions or DNA hybridization, or in general, any recognition reaction between a receptor and its ligand, is particularly attractive [1–6]. Among these, electrochemical impedance spectroscopy (EIS) represents one of the most powerful methods, especially in terms of simplicity (no labeling is needed) and of its ability to be more easily integrated into multi-array diagnostic tools or microprocessor-controlled implanted sensors [1,2].

Several instruments based on small-signal ac admittance measurements such as the LCR-meter [7–9], impedance analyzer [5], lock-in amplifiers [3,4,10,11] and frequency response analyzers (FRAs) [12–17] have been used to monitor the interactions between biomolecules. The last two approaches are the most widely used and bring inherent advantages and disadvantages. Impedance systems based on lock-in amplifiers are very sensitive, can effectively remove background noise, minimize harmonic distortions and are relatively cost effective; on the other hand, it is difficult to be used for stand-alone measurements, measurements are somewhat slow, and they cannot be performed over a wide frequency range. Impedance systems based on FRA, provide fast analysis over a wide frequency range, remove harmonic distortions and dc components and can be easily fully automated. Limited sensitivity and background removal as well as their relative high cost are certain disadvantages associated with FRA-based measuring systems [18]. Berggren and Johansson evaluated capacitance changes from the transient current response after the application of a potentiostatic step [19]. In this approach, the capacitance
of the sensor was extracted from the time constant of the exponential fit to the current versus time trace.

Among the commercially available instruments, AUTOLAB/FRA2 (Eco Chemie, The Netherlands), IM6e (BAS-Zahner, USA-Germany), FRA1260/1255 (Solartron Analytical, UK), PARSTAT 2273 (Princeton Applied Research, USA), Voltalab (Radiometer Analytical, France) are the most cited analyzers, and they use digital-to-analog converters for generating the excitation waveform; alternatively, FC350 analyzer (Gamry Instruments, USA) uses a digital controller to generate the sinusoidal current signal. More information about the marketplace and the abilities of other commercial analyzers can be found in the product review of Smith and Hinson-Smith [20].

The physical parameters that have been used so far, as a measure of the analyte concentration, include, capacitance, charge-transfer resistance and impedance. Capacitance measurements can be performed using a lock-in amplifier, and in the case of an RC circuit, capacitance can be also calculated as $1/Z\omega$, using a FRA-based analyzer; however, this type of circuits is not common in practice, since the immobilization of biomolecules introduces resistance elements. Charge transfer resistance has been successfully used as a measure of the analyte concentration [6, 14]. The time consuming process of obtaining $Z''=f(Z')$ spectra (Nyquist plots) over a wide frequency range is the main disadvantage of this approach. Alternatively, impedance ($Z$, $Z'$ or the magnitude $|Z|$) values can be used as a measure of the analyte concentration. This option provides fast measurements and the ability to monitor the kinetics of the immunocoupling [3, 13]. Another option for the calculation of the above parameters is the fitting of equations associated to an equivalent circuit to the experimental data using commercial software [12, 13, 17]. However, assuming that no equivalent circuit can be guaranteed to be unique, simulated values of the discrete elements are mostly used for supporting the validity of a given model and not for analytical purposes.

In the present paper, we report the use of a homemade electronic device, hereafter referred as ‘Multipulser’, for monitoring interactions between biomolecules that may change the capacitance of an electrode. Multipulser can be used as a stand-alone, low-cost yet effective alternative monitoring device instead of the other well established instruments mentioned above. The operation of Multipulser is based on the repetitive charging of the electrochemical cell capacitance by applying a predetermined number of short-duration, low-amplitude voltage pulses (perturbation pulses). All packets of charge are accumulated in an analog integrator whose output voltage is proportional to the cell capacitance.

The perturbation pulse approach used by Multipulser is similar to the one used by Berggren and Johansson [19] mentioned before, but instead of fast sampling the exponentially decaying current, the whole area under the decaying current (i.e. the electric charge) is measured, an approach that simplifies considerably the required instrumentation.

Multipulser was tested with two different electrode assemblies; one based on thiol self-assembled monolayers (SAMs) on gold (Au-electrode assembly, Scheme 1, A) and the other based on a Ti/TiO$_2$ semiconductor electrode (Ti/TiO$_2$-electrode assembly, Scheme 1, B), both of them containing covalently linked avidin. The binding of biotinylated dextran on their surface served as the model system. These electrode assemblies respond differently to the avidin–biotinylated-dextran complex formation. Measurements conducted in parallel with a commercial frequency response analyzer gave reaction patterns in agreement with those observed using Multipulser. In addition, the bipolar potential perturbation used by Multipulser, revealed a peculiar pulse-polarity depen-
dent behavior in the case of the Ti/TiO₂-electrode assembly, which, to the best of our knowledge, has never before been reported.

2. Hardware and principle of operation

The principle of operation of the Multipulser is depicted in Fig. 1. Assuming that the dielectric layer (insulating layer + biorecognition layer) of the sensing electrode behaves as a perfect isolator (i.e. the ohmic resistance of the dielectric layer is infinite) then the electrically equivalent circuit of the cell can be simply approximated by the resistor \( R_{\text{cell}} \) and the capacitor \( C_{\text{cell}} \) in series as it is shown in Fig. 1. \( R_{\text{cell}} \) typically the resistance of the bulk electrolyte solution and the reference (or quasireference) electrode used as a counter electrode. Since the capacitance of the counter electrode is much larger than the capacitance of the sensing electrode, \( C_{\text{cell}} \) represents only the sought-for capacitance of the latter.

The perturbation pulses of height \( V_p \) (typically: \( 5\text{mV} \leq V_p \leq 100\text{mV} \)) and of short duration \( T \) (typically: \( 0.1\text{ms} \leq T \leq 20\text{ms} \)) are applied to one electrode of the cell (Fig. 1, inset a). The second electrode of the cell is connected to an inverting amplifier consisting of the operational amplifier OA1, the input resistor \( R_{\text{in},1} \) and the feedback resistor \( R_f \).

According to the basic operating principles of operational amplifiers, their output voltage acquires a value that through the negative feedback element equates the voltages applied to both inputs. Since the non-inverting input is grounded, the voltage at the inverting input of OA1 remains always zero although this input is not directly connected to the circuit ground, i.e. it acts as virtual ground, denoted by the ground symbol in parenthesis shown in Fig. 1. Upon applying a perturbation pulse a purely capacitive current flows through the cell charging \( C_{\text{cell}} \). This current is limited by the sum resistance \( R_{\text{cell}} + R_{\text{in},1} \). Assuming that \( R_{\text{in},1} \gg R_{\text{cell}} \), then \( R_{\text{in},1} \) can be neglected. Hence, this current decays exponentially from a peak value \( i_p = V_p / R_{\text{in},1} \) to zero during the pulse life, according to equation:

\[
i_{\text{cell}} = \frac{V_p}{R_{\text{in},1}} \exp \left( -\frac{t}{R_{\text{in},1}C_{\text{cell}}} \right)
\]

After the termination of the perturbation pulse, \( C_{\text{cell}} \) is discharged giving rise to an exponentially decaying current of opposite direction (Fig. 1, inset b).

Since the input current of operational amplifier is practically zero, \( i_{\text{cell}} \) flows through the feedback resistor \( R_f \) of the inverting amplifier, and in order to maintain the inverting input at zero voltage, OA1 generates an output voltage \( v_{o,1} \) given by the equation:

\[
v_{o,1} = -i_{\text{cell}} R_f = \frac{V_p R_f}{R_{\text{in},1}} \exp \left( -\frac{t}{R_{\text{in},1}C_{\text{cell}}} \right)
\]

This voltage is fed through a fast solid state switch SW1 to a typical analog integrator consisting of the operational amplifier OA2, the input resistor \( R_{\text{in},2} \) and the capacitor \( C \) as the negative feedback element. In synchronization with the perturbation pulse, switch SW1 is in position 1 when \( v_{o,1} = V_p \) and in position 2 (grounded) when \( v_{o,1} = 0 \). Thus, only the exponentially decaying voltage that is associated with the charging current is integrated, whereas the opposite direction decaying voltage associated to the discharging current is effectively clipped (Fig. 1, inset c).

Switch SW2 (when in position 2) resets the integrator output voltage to zero. The output voltage \( v_o \) of the integrator is generally given by the equation:

\[
v_o = -\frac{1}{R_{\text{in},2}C} \int_{0}^{t} v_{o,2}(t) dt
\]

Since it is \( v_{o,2} = v_{o,1} \) during the perturbation pulse and \( v_{o,2} = 0 \) otherwise, Eqs. (2) and (3) can be combined to the
The corresponding

As it is obvious from Fig. 2 a relatively wide range of acceptable proportionality between \( v_o \) output and \( C_{\text{cell}} \) is expected, when \( \tau \ll T \), indicating the expected shape of the working curve (integrator output vs. the sensing electrode capacitance) over an extended \( T/T \) range under the assumptions given in text.

Thus, for \( \tau = R_{\text{in}} C_{\text{cell}} = 0.3 T \) the expected deviation from the proportionality assumed by Eq. (8) is less than 4%, since \( 1 - \exp(-10.3) = 0.964 \).

The user can adjust only the following measurement parameters: the pulse height \( V_p \), the number of pulses \( N \) and the pulse width \( T \). All other parameters (component values) are fixed as follows: \( R_{\text{in},1} = 10 \, \Omega, R_{1} = 10 \, \Omega, R_{2} = 330 \, \Omega \), and \( C = 1 \, \mu F \).

2.1. Operating modes

Multipulser features three user selectable operating modes each one characterized by its own particular shape of the applied perturbation pulses. The shape of each period, corresponding to each mode, is shown in Fig. 3. Mode 1 represents

0.3 T

0.5 T

1 T

1.5 T

2.5 T

Fig. 2. Dimensionless plot showing the normalized voltage output \( (v_o/\Delta v_o) \) vs. the ratio of cell time constant over pulse width \( (\tau/T) \), indicating the expected shape of the working curve (integrator output vs. the sensing electrode capacitance) over an extended \( T/T \) range under the assumptions given in text.
the typical operation of Multipulser as it was previously described. Each period \( P \) consists of two similar half-periods (to maintain a uniformity of period definition with the other two modes) and it is composed of two perturbation pulses of height \( V_p \) of the same polarity, each of width \( T = P/8 \). The relaxation sub-period (zero voltage) between two consecutive pulses is \( 3P/8 \).

Mode 2 is similar to mode 1 but the two perturbation pulses have opposite polarities (bipolar perturbation). By using mode 2, possible polarization phenomena are prevented and this should be the preferable mode particularly when rather high \( V_p \) values are going to be applied. In the absence of polarization phenomena, modes 1 and 2 should yield the same output signal. In order to integrate the charge from both pulses, during the application of the second pulse an inverting amplifier of gain \( \times (-1) \) is inserted in the signal path prior to the integrator, thus both packets of charge arrive to the integrator having the same sign.

Mode 3 came as a necessity for electrodes characterized by a dielectric layer of relatively low resistance. In these cases, contrary to what is expected with dielectric layers of near-infinite resistance (Fig. 3, mode 3, \( i_{cell} \) curve a), \( i_{cell} \) never decays to zero (Fig. 3, mode 3, \( i_{cell} \) curve b), due to the constant (dc) component of non-capacitive current flowing through the cell. Mode 3 reduces or eliminates the contribution of this dc current component to the measured signal. For preventing possible polarization phenomena, a bipolar square waveform is also used in mode 3, hence there are no relaxation sub-periods. Therefore, after each polarity transition, the cell current is given enough time to decay to the value determined by the resistance of the dielectric layer. The charge corresponding to the last quarter of each half-period is always introduced to the integrator with the opposite sign to that of the first quarter, effectively correcting the integrated charge for this constant current background. This is achieved by inserting the inverting amplifier of gain \( \times (-1) \) before the integrator at the appropriate period segment, in order to maintain a constant sign of the integrated packet of charge as in mode 2.

In the case of a near-infinite dielectric resistance, mode 3 yields almost the double output signal compared to that obtained when modes 1 and 2 are used. This is due to the fact that the voltage transitions taking place in mode 3 (+\( V_p \) to −\( V_p \)) are twice those used in modes 1 and 2 (+\( V_p \) or −\( V_p \) to 0).

### 2.2 Block diagram of the Multipulser

The block diagram of the Multipulser electronic circuit is shown in Fig. 4. The main units of the analog part of this circuit are:

(i) The variable voltage source consisting of a constant voltage source, a voltage divider and a follower amplifier (not shown in Fig. 4). A multturn potentiometer acting...
as a voltage divider allows the user to select any height \((V_p, \text{Fig. 3})\) of the perturbation pulse up to 100 mV. The follower amplifiers provide loading of the voltage divider and a low output resistance.

(ii) The input (inverting) amplifier whose primary function is to act as a constant ohmic resistance path to virtual ground for the charging/discharging current from the cell.

(iii) The integrator that essentially collects all charge packets and develops an output voltage signal proportional to the total accumulated charge. A front-panel digital voltmeter is used for displaying this signal to the user.

(iv) Two inverting amplifiers with gain set exactly to \(52\) ensuring extremely low bias current and easy drift suppression for the analog integrator.

A quad SPDT CMOS analog switch MAX333 (Maxim) (SSW1-4) provides the necessary fast switching actions. SSW1 selects the polarity of the perturbation voltage, SSW2 applies the selected perturbation voltage or a zero (relaxation) voltage to the cell, SSW3 selects the polarity of the response signal sent to the integrator, and SSW4 selects the segment of the response signal to be integrated. A miniature reed relay RRL is used to reset the integrator at the beginning of each measurement sequence.

The digital part of the circuit consists of a RISC 8-bit microcontroller AT90S8515 operating at 8 MHz (Atmel Co., San Jose, CA.). Its main task is to coordinate and provide the necessary control signals (according to the selected mode) to all digital switches and to the reed relay. The user interface consists of a front-panel digital keypad passing \((1–3); \text{(ii) number \((N)\) of issued periods \((1–65535); \text{(iii) full width \((1–65535 ms)\). There are also some single-key commands (‘RUN’, ‘ABORT’, ‘CLEAR’) to facilitate operation. Additional instructions have been provided to assist the balancing of all inverting amplifiers, and the minimization of the drift of the integrator during the periodic re-alignments of the Multipulser. Perfect balancing of all amplifiers is indicated when a dummy cell measured in modes 1 and 2 yields the same signal output. A standard dot-matrix LCD 2 × 16 characters display is used to facilitate the keying of parameters, and for displaying the current settings (mode, \(N\) and \(P\)) and the actual operational status.

3. Experimental

Chemicals. Avidin (egg white, lyophilized, MW 66 kDa) and biotinylated dextran (MW 70 kDa) were purchased from Calbiochem (La Jolla, CA) and Fluka (Buchs SG, Switzerland), respectively. 16-Mercaptohexadecanoic acid (HSR15 COOH), 3-aminopropyltrimethoxysilane (APTES), N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) and glutaraldehyde (grade II, 25%) were obtained from Sigma (St. Louis, USA) and used as received. 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), glycine and bovine serum albumin (BSA) were obtained from Aldrich (Gillingham, Germany). All reagents used were of analytical grade. Double distilled water (DDW) was used throughout. All solutions were prepared in a 50 mM HEPES pH 8 buffer solution, unless otherwise stated.

3.1. Apparatus

Electrochemical impedance spectroscopy (EIS) experiments were performed with the Autolab Analyzer (PGSTAT12/FRA2, Ecos Chemie, The Netherlands) in a one-compartment three-electrode voltammetric cell under quiescent conditions. The Au- and Ti/TiO2-electrode assemblies were used as working electrodes. The reference electrode was a Ag/AgCl/3 M KCl (BAS, IN, USA) and a platinum wire served as the auxiliary electrode. The impedance spectra were recorded over the frequency range \(10^{-1}\) to \(10^{3}\) Hz by using a sinusoidal excitation signal. The applied potential was 0.050 V. Excitation amplitude of 10 mV was used throughout. Measurements with the Multipulser were made in the same cell at a 2-electrode mode (working and auxiliary) under mild stirring (100 rpm).

3.2. Electrodes fabrication and modification

Gold and titanium disc electrodes of 2 mm diameter active surface were both constructed by using the commercial kit EasyCon (EasyCon Hellas, provided by Eco Chemie). Gold electrodes were cleaned as follows: gritting with a 4000-grit emery paper, sonication for 1 min in DDW, polishing with extra fine alumina, sonication 1 min in ethanol and finally drying with argon. The electrodes were immersed in a 2.0 mM HSR15-COOH (Note: Handling must be carried out in a hood wearing rubber gloves) in ethanol overnight, sonicated for 1 min in ethanol to remove excess of non-bound thiol and then rinsed thoroughly with ethanol. Carboxyl ends were activated and coupled with avidin’s carboxyl and/or amine ends by incubating the carboxyl terminated gold surfaces in a mixture of 0.1 mL of 1 mg/mL avidin in PBS pH 7.4 and 0.5 mL of 0.15 M EDC in 10 mM NaH2PO4, pH 4.0 for at least 12 h at +4 °C.

Ti/TiO2-electrodes were anodically formed at 30 V in 2 M H2SO4 for 1 h. Passive films of titania (TiO2) were hydroxylated in 0.5 M NaOH for 5 min and then silanized in 10% APTES (Note: Handling must be carried out in a hood wearing rubber gloves) in dry toluene for 30 min. After incubation at 90 °C for 3 h, the silanized surfaces were activated for 2 h with 2.5% glutaraldehyde. Formyl terminated surfaces were incubated overnight at 4 °C in 1 mg/mL avidin.
Both avidin-modified architectures were incubated in 0.025 M glycine (pH 7) for 1 h to ensure totally blocked formyl ends, thoroughly rinsed with HEPES and then incubated overnight with 1, 10 or 100 g/mL solutions of biotinylated dextran at room temperature.

4. Results and discussion

As mentioned above, the performance of the Multipulser was tested in two different electrode assemblies, using the avidin–biotinylated dextran complex formation as a pilot system. These electrode assemblies were selected because the results obtained with the commercial FRA system indicated that the receptor-ligand binding resulted into different direction of capacitance change for each electrode assembly.

4.1. Performance of FRA-based analyzer and Multipulser with a thiol/avidin/biotinylated-dextran architecture

Electrochemical impedance spectra (Nyquist and Bode plots) were obtained over a wide range of frequencies ($10^{-1}$ to $10^4$ Hz) with Au-electrode assemblies unexposed and exposed to different analyte (biotinylated-dextran) solutions. The obtained plots (Fig. 5) indicate that $Z'$ and $Z''$ (and $|Z|$) of the impedance increase after exposure to the analyte.

At high frequencies ($>10^4$ Hz) the curves do not depend strongly on the analyte concentration, demonstrating that at high frequencies the impedance is (mostly) sensitive to the composition of the buffer solution. Increase of the impedance (Table 1) as well as the increase of $R_q$ (total electron-transfer resistance at the electrode/electrolyte interface) values (Fig. 5) can be effectively used as a measure of the extent of the biomolecules coupling reaction. Increase of $R_q$ can be attributed to the formation of the avidin–biotinylated dextran complex, which retards the interfacial electron-transfer kinetics at the electrode–electrolyte interface.

Nyquist and Bode plots are indicative of a typical Randles circuit, i.e. $R_	ext{ohm}(R_qC_{bc})$. $R_	ext{ohm}$ corresponds to the ohmic resistance of the bulk solution and depends on the ionic strength of the buffer used. $R_q$ is dependent upon structural and electrical features of the various coatings interposed between the transducer and the bulk solution, i.e. self-assembled mono-

![Figure 5](image)

Table 1: Impedance ($Z'$, kΩ; imaginary) values of the Au/HSR$_2$COOH/avidin electrodes measured before and after their incubation with different biotinylated-dextran concentrations.

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>Biotinylated dextran (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>74.5</td>
</tr>
<tr>
<td>10</td>
<td>10.5</td>
</tr>
<tr>
<td>100</td>
<td>1.5</td>
</tr>
<tr>
<td>1000</td>
<td>0.22</td>
</tr>
<tr>
<td>10000</td>
<td>0.035</td>
</tr>
</tbody>
</table>

$E=0.050$ V, $r_m=10$ mV.

The response of the Multipulser for the same system, under similar experimental conditions is illustrated in Fig. 6 A. The measured signal is decreasing upon the addition of the analyte (point 1). Further decrease of the overall charge was recorded after rinsing of the sensor with the working buffer solution (point 2), followed by a slight increase of the recorded signal, attributable to partial desorption of the analyte from the immobilized avidin layer (point 3), upon standing of the Au-electrode assembly in the working buffer solution. Further
Fig. 6. Plots of Multipulser signal output vs. time. (A) Au-electrode assembly; (1) m o d e 2 (N = 300, P = 300 ms); (2) m o d e 3 (N = 150, P = 300 ms); (1) injection of 10 μg/mL analyte; (2) rinse with the working buffer; (3) injection of 100 μg/mL analyte; (4) rinse with the working buffer. (B) Ti/TiO2-electrode assembly; modes (1) and (2). (N = 2000, P = 50 ms); (1) injection of 10 μg/mL analyte; (2) rinse with the working buffer.

decrease of the signal upon re-incubation of the sensor in a higher concentration of the target analyte was recorded. Signal profile in point 4 can be explained as in case of point 2.

The results obtained by the Multipulser compare well with those received by the commercial analyzer. In both cases, the binding of the analyte by the immobilized avidin resulted into an impedance increase indicated (in the case of Multipulser) by a decrease of the output signal, i.e. decrease of the overall capacitance. The results obtained by Multipulser in either mode (2 or 3) are nearly identical in terms of relative change of the output signal, indicating an almost ideal capacitive behavior (high dielectric resistance).

Although a signal output decrease of about 30% was obtained after 16 h, much shorter (0.5–1 h) measurements of kinetic nature and more suitable for analytical purposes are feasible, as it is indicated by the signal change immediately after the analyte addition (Fig. 6A, inset).

4.2. Performance of FRA-based analyzer and Multipulser with Ti/TiO2/aminosilane/avidin/biotinylated-dextran architecture

The Nyquist plot in Fig. 7A, shows a behavior similar to that of Au-electrode assembly illustrated in Fig. 5, regarding the behavior of the system in the low frequency region, i.e. charge transfer resistance is increasing after the exposure to the analyte. However, data in Table 2, shows that impedance values are decreasing upon the addition of the analyte, except in the range of very low frequency <10Hz. Frequency dependent alteration of the algebraic value of ΔZ has also been observed by other groups and can be attributed to the domination of each of the discrete elements included in the equivalent circuit at the specific frequency range [13]. This behavior is

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>Z′ (Ω)</th>
<th>Z″ (Ω)</th>
<th>Z (Ω)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti/TiO2/aminosilane/avidin electrode</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10000</td>
<td>1.01</td>
<td>0.91</td>
<td>1.365</td>
</tr>
<tr>
<td>1000</td>
<td>3.2</td>
<td>5.3</td>
<td>6.1</td>
</tr>
<tr>
<td>100</td>
<td>18.3</td>
<td>17.9</td>
<td>25.6</td>
</tr>
<tr>
<td>10</td>
<td>54.4</td>
<td>33.5</td>
<td>63.9</td>
</tr>
<tr>
<td>1</td>
<td>114.1</td>
<td>29.4</td>
<td>117.8</td>
</tr>
<tr>
<td>After incubation in 1 μg/mL biotinylated dextran</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10000</td>
<td>0.99</td>
<td>0.67</td>
<td>1.193</td>
</tr>
<tr>
<td>1000</td>
<td>2.7</td>
<td>3.2</td>
<td>4.2</td>
</tr>
<tr>
<td>100</td>
<td>9.9</td>
<td>10.1</td>
<td>14.2</td>
</tr>
<tr>
<td>10</td>
<td>30.6</td>
<td>37.2</td>
<td>48.2</td>
</tr>
<tr>
<td>1</td>
<td>134.9</td>
<td>120.0</td>
<td>180.6</td>
</tr>
</tbody>
</table>

E = 0.050 V, rms = 10 mV.
clearly illustrated in Bode plots in Fig. 7B (overall impedance is decreased over the frequency range 5–10000 Hz, and increased at frequency lower than 5 Hz), thus giving more insight for the behavior of the system. In contrast to the behavior showed above for the Au-electrode assemblies, the Ti/TiO₂-electrode assemblies exhibit two time constants. The one at high frequency region can be attributed to the stannia/aminosilane layer and the other, at the lower frequency region, to the avidin–biotinylated-dextran interactions. Since the length scales associated with the diffuse layers and the molecular layer are comparable to one another, the physical structure of the interface is not atomically sharp. In accordance to the findings reported by Lasseter et al. [12] binding of biotinylated dextran to avidin changes both layers. A series of experiments has been performed with Ti/TiO₂ electrodes coming from different batches and different anodization times (different thickness of the oxide layers and different allotropic forms of the developed oxide). Detailed data for each step of the architecture build-up as well as for a number of different concentrations of biotinylated dextran is given elsewhere [22].

The response of the Multipulser for the same system, under similar experimental conditions is illustrated in Fig. 6B, and it is fully consistent with the impedance profile recorded by the commercial FRA analyzer, as the measuring signal is increasing upon the addition of the analyte (point 1). As it can be seen in Fig. 6B, signal profiles in both applied potential waveforms (modes 2 and 3) are almost identical; however, the relative signal change immediately after point 1 is significantly different. The much higher relative change obtained using Multipulser in mode 3 indicates a significantly low dielectric resistance contrary to what was observed in the case of the Au-electrode assembly. Since mode 3 provides a correction for the constant current component, the relative signal change is more closely associated with the capacitance change during the binding reaction.

The capacitance increase observed with the Ti/TiO₂-electrode assemblies (see above) can be attributed to the formation of a new composite material, that is, aminosilane/avidin/biotinylated dextran, which has a higher (observed) dielectric constant [22].

More insight to the different performance of the tested electrode assemblies, as both the commercial FRA-based analyzer and the Multipulser have recorded it, can be obtained by observing the current pattern associated with the potential waveforms provided by modes 2 and 3. Multipulser provides signal monitoring test-points at various critical points of the analog circuit. The voltage applied to the integrator unit is indicative of the cell current (after the sign change provided by the switches/inverter networks). A completely pulse-polarity independent behavior is manifested by similar current decay patterns in both half-periods, each one corresponding to opposite polarity perturbation potential in modes 2 and 3. This behavior is perfectly met with dummy cells and also in the case of the Au-electrode assemblies. In the case of the Ti/TiO₂-electrode assemblies, a characteristically strongly polarity-dependent behavior was consistently observed. Typical current patterns are shown in Fig. 8. In these patterns it is also obvious that the cell current does not decay to zero indicating the presence of a constant current component that makes mode 3 preferable over mode 2 for reaction monitoring.

Although this behavior requires further experimental study, it can be attributed, considering the time scale, to alternating adsorption–desorption cycles of ions on and from the hydrous TiO₂ layer; that may be followed by periodic blocking of the conductivity channels resulting thus into periodic alterations of its ohmic resistance.

4.3. Aspects related to the operation of Multipulser

The relatively low input impedance of the input stage \( (R_{in} = 10 \, \text{k} \Omega) \) assures a low noise pick-up, thus no shielded cables are required for the connection of the cell electrodes to the device and the length of cables used is not of major concern. In addition, the polarity of this connection is irrelevant, i.e. the same output signal is obtained regardless of which electrode (working or auxiliary electrode) is connected to the input of the inverting amplifier. A difference may be observed only when mode 1 is used and polarity dependent phenomena occur, as those described before, and this property can serve as an efficient diagnostic tool for the existence of such phenomena.

The repeatability of the indications provided by the Multipulser is about 0.1–0.2% when dummy cells are used. Similarly stable indications over extended time periods were ob-
tained when well known as stable electrode assemblies were tested (e.g. Au/l-dodecanethiol SAMs). Potentiostatic control commonly used in impedimetric measurements does not seem to be a necessity and the output signal seems to be independent of the positioning of the electrodes in the cell, the existing hydrodynamic conditions and of the ionic strength of the measured solutions, provided that $R_{\text{m}}$ is kept much lower than the input resistance of the inverting amplifier ($R_{\text{in}}$).

5. Conclusions

The change of the recorded signal in the electrochemical monitoring of a binding reaction is greatly affected by a number of parameters, such as the morphological (thickness, roughness) and electrical features of the insulating layer, the chemical nature of it (polymer, metal oxide/silane, thiol-based SAMs) and the dielectric character of the bilayer. Multipulser proves to be adequate for the monitoring receptor–ligand type interactions, being a cost-effective alternative approach to impedimetric measurements. Whereas Multipulser does not provide the wealth of information provided by FRA, it seems ideal for monitoring kinetically electrode capacitance effecting processes for purely analytical purposes. The ability to provide information regarding the existence of any polarity-dependent phenomena in the tested electrode assembly is certainly an additional advantage.

Although Multipulser has been designed with the prospect of developing a handheld device capable of a stand-alone operation, $I/O$ lines of the microcontroller have been used to provide the control of a simple multiplexer unit that connects the Multipulser to up to eight different cells, in order to obtain, virtually simultaneously, more measurements (i.e. standards + unknowns) over the same time period.

Acknowledgments

The financial support of EC (Project IMAGEMO; QLK3-CT-2002-02141) is gratefully acknowledged. Authors wish to thank, Dr. T. Frelink (Eco Chemie) and Dr. P. Millner (University of Leeds), for fruitful discussions.

References


Biographies

Spiros D. Bolis received both his first degree in chemistry and his MSc from the University of Athens while now pursues his PhD in the same institution. His main research interests involve analytical electrochemistry and chemical instrumentation. He is also involved in flow and sequential injection analysis projects. His task in the IMAGEMO project, under which the current work was carried out, is the design, implementation and evaluation of the charge accumulating analyser for impedimetric determination of GMOs.

Panagiota C. Charalambous is a PhD candidate at the University of Athens working on electrochemical impedance spectroscopy and impedimetric biosensors. She has a first degree in chemistry and an MSc in analytical chemistry from the University of Athens. In the past, she has been involved in flow injection analysis using chemiluminescence for the determination of various environmental pollutants.

Constantinos E. Efstathiou received his BSc and PhD degrees in chemistry from the University of Athens, Greece, in 1971 and 1976, respectively. He is professor of chemistry and he is currently the director of the Analytical Chemistry Laboratory of the Chemistry Department in the University of Athens. He has 80 scientific publications on topics including electroanalytical techniques (ion-selective electrodes, stripping voltammetric techniques, modified electrodes), kinetic methods of analysis, chemometrics and the application of microcomputers in the automation of various analytical techniques. He is author and co-author of books on general analytical chemistry, electroanalytical techniques, chemical instrumentation and microcomputers and chemometrics.

Aikaterini G. Mantzila received her BSc degree in chemistry from the University of Ioannina (Greece) in 2005. She is currently undertaking her PhD studies in the development of oxide-based capacitance immunosensors.

Constantina A. Malamou received her BSc degree in chemistry from the University of Ioannina (Greece) in 2003. She is currently undertaking her PhD studies in the development of bion-modified gold-based capacitance immunosensors.

Mamas I. Prodromidis received his BSc and PhD degrees in chemistry from the University of Ioannina, Greece in 1993 and 1997, respectively. He is a lecturer in the Section of Analytical Chemistry and his research interests include the development of analytical methods based on chemical or biochemical amperometric sensors, capacitance immunosensors, new materials for electroanalytical applications, fabrication of screen-printed electrodes, all-solid-state potentiometric electrodes and instrumentation. He has 40 publications and one patent. The latter had led to the establishment of a spin-off company on the construction of voltammetric sensors. He is currently coordinating a European project and he has also involved in various national and European projects.