Impedance Measurements as a Tool for the Detection of Endocrine Disrupting Chemicals

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Abstract-**The growing awareness worldwide over the deleterious effects of endocrine disruptors on human health has created a need for screening systems to detect xenoestrogens, a diverse group of chemicals that mimic estrogenic actions and are assumed to decrease mail fertility and induce hormone related cancers in females. Here we describe a novel, class-selective detector that uses fast impedance measurements to monitor the binding of estrogen and xenoestrogens to a native estrogen receptor. We embedded the receptor in synthetic lipid bilayers attached to gold electrodes. The lipid bilayers serve as electrical circuits constructed of resistors and a capacitors. Estrogen binding to the receptor-modified electrode is immediately followed by conformational changes in the lipid layer, leading to alterations of the electrical circuit components that are detected by fast impedance measurements. The electrochemical system enabled characterization of changes in the bilayer structure and quantification of estrogen binding to the receptor. To assess the effectiveness of the method for detecting estrogenic chemicals, we chose two classes of xenoestrogens: bisphenol-A, a synthetic xenoestrogen, and genistein, a phytoestrogen. This system is highly sensitive and amenable to use on-site, providing an efficient and economic tool for measuring minuscule amounts of endocrine disrupting chemicals in environmental or human samples.**

Keywords; **Endocrinic disrupters, xenoestrogens, impedance, biosensor**

I. INTRODUCTION

Estrogen is a hormone produced mainly in the ovaries that is essential for the normal development, maturation, and function of the reproductive tract in females. Additional small amounts are secreted by the adrenal glands and by the male testes. Estrogen regulates cellular events through a specific intracellular receptor that functions as a ligand-inducible transcription factor. Hormone binding to the estrogen receptor is followed by a series of events that include dissociation from heat shock proteins, dimerization and binding to DNA at an estrogen response element> @ The 3-D structure of the ligand binding domain has been solved> @

Environmental estrogens, as well as endogenous ovarian estrogens, are thought to initiate their physiological actions in target tissue via interactions with a nuclear receptor system. Receptor overactivation, followed by overexpression of the hormone, results in the feminization of wild life like fish, alligators, rats, and turtles; and may contribute to the increasing incidence of breast and testicular cancer in humans> @

Xenoestrogens are naturally occurring and anthropogenic chemicals possessing estrogenic activity. Deriving the toxic potential from their structures is difficult because of the wide variety of xenoestrogenic compounds. The structural diversity of the xenoestrogens has confounded attempts to measure the estrogenic potential of samples of estrogenic and xenoestrogenic compounds using conventional chemical analyses> @ Monitoring and evaluating the effects of xenoestrogens require new technologies that are capable of screening various chemicals in the field> @Because of their cost, complexity, and ethical concerns, traditional bioassays are inadequate for the large-scale screening of environmental chemicals in an effort to preclude their release.

The ability to detect xenoestrogens in environmental samples is a goal that is being actively pursued by bioorganic chemists. Environmental analysis of xenoestrogens should consist of two parts. First, a good screening method should detect all classes of xenoestrogens and measure a large number of potentially contaminated sites. Positive samples should then be re-analyzed with current analytical techniques to identify the compound(s) responsible for the estrogenic effect. Such analyses can be executed by using LC, GC, MS, and/or NMR> @

Since the early 1960s, bilayer lipid membranes (BLMs) have been the most widely used experimental models of biomembranes> @The major constituents of cell membranes are phospholipids, along with proteins, carbohydrates, and their complexes, which spontaneously self-assemble into lipid bilayers. Membranes supported on solids are of practical and scientific interest for several reasons: (1) they enable the biofunctionalization of inorganic solids (semiconductors, gold-covered surfaces, optoelectronic devices) and polymeric materials; and (2) they provide a natural environment for immobilizing proteins like hormone receptors and antibodies under non-denaturating conditions and in a well-defined orientation> @

Biosensors combine a biological recognition mechanism with a physical transduction technique, providing a class of inexpensive, portable instruments that permit sophisticated analytical measurements to be undertaken rapidly at decentralized locations> @In the present study, we used such a technique to study events following the binding of estrogen or xenoestrogen to a native estrogen receptor embedded within a synthetic lipid bilayer membrane, self-assembled onto a gold electrode. Estrogen or xenoestrogen binding to the receptor-modified electrode was immediately followed by conformational changes in the lipid layer, leading to alterations of its electrical properties. Fast impedance measurements revealed these alterations before and after binding.

II. EXPERIMENTAL SECTION

1). *Materials:* Synthetic L-phosphatidylcholine,-oleoyl--palmitoyl (PCOP) and Decanthiol were purchased from Sigma (99%, TLC). Estrogen receptor-? human recombinant, specific activity; 5000 units/mg protein was purchased from Calbiochem. One unit is defined as the amount of enzyme that will bind 1.0 pmol of ^{3}H-estradiol in 2 h at 22 °C, pH=7.5. Purity ≥80% by SDS-PAGE, M.W. 66,000. Soluble estrogen (17-beta-estradiol), bisphenol-A and genisteine from soybean (high purity grade) were

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Abstract

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purchased from Sigma. Potassium hexacyanoferrat(II) and potassium hexacyanoferrat(III) (AR) were purchased from Fluka. All other inorganic chemicals were of analytical grade.

2). *The Electrochemical Setup:* A 50 mL three-electrodes electrochemical cell was used in all measurements. Saturated calomel electrode (SCE) was used as a reference electrode, (Radiometer K401, Denmark), platinum wire as a counter electrode and a gold electrode (0.04 cm^2, Bioanalytical Systems, USA) as the working electrode. Before use the gold electrodes were polished with alumina 0.5 micron and then with alumina 0.03 micron washed with double-distilled water. The electrodes were then immersed in a sonicator bath for 20 min, followed by washing in double-distilled water. The last procedure was repeated three times. All electrochemical experiments were conducted in buffer solutions containing 0.1 M phosphate buffer (pH 7.4) and 0.1 M KCl (PBS).

3). *Construction of the Bilayer and Receptor Binding:* Six clean gold electrodes were placed in ethanol solution of 10 mM decanthiol for 16 h to produce the first layer. Next, a solution of 0.2 mM PCOP was sonicated (Misonix Microso^{TM} ultrasonic cell disrupter, output power of 20 watts rms) for 30 sec to produce liposomes. The gold electrodes were then placed in the 700 ? L liposome solution for 2 h to produce the bilayers. Finally, 5? L containing 30 pmol of human estrogen receptor in PBS were applied directly onto the bilayer electrodes surface. The electrodes were then protected from dust by plastic covers. After 40 min, the electrodes were washed with PBS to remove the unbound receptor. Each step of the biolayer construction was examined by applying the methods described below. After exposure, the modified electrode to the ligand was washed carefully with 1 M NaOH and then with 1 M HCl [24].

4). *Electrochemical Measurements:* Alternating current (AC) impedance measurements were conducted using the BAS100W, Electrochemical Analyzer (Bioanalytical Systems, USA.). Galvanostatic measurements were performed using the 273 EG&G PAR potentiostat galvanostat and EG&G 270 software.

5). *AC Impedance*: The modified electrode described above, was placed in the electrochemical cell containing PBS and 10 mM $Fe(CN)_6^{4}$/$Fe(CN)_6^{3}$. Measurements were done at 180 mV vs SCE. After each stage described above, an alternating voltage signal of ±10 mV in the frequency range of 0.01 to 1000 Hz was applied, and the resulting AC was followed. After the AC data were analyzed, Nyquist plots were drawn.

6). *Galvanostatic Measurements:* Galvanostatic measurements were conducted in solutions containing 0.1 M PBS (no redox couple added). The electrochemical cell was stabilized by holding at zero current, followed by a step of 1 ? A for 0.1 to 2 seconds. The potential change was monitored and analyzed.

III. RESULTS AND DISCUSSION

A. AC Impedance

Figure 1 presents four Nyquist plots obtained for the consecutive stages of the sensor constructions e.g., (a) the

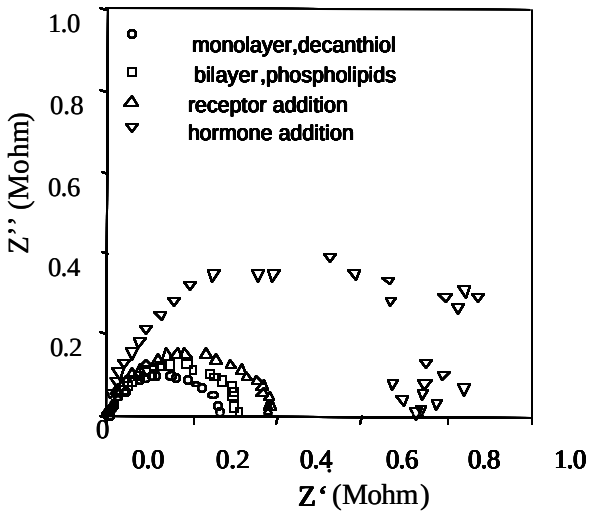

Fig 1: Nyquist plots for biolayer constructions as measured by AC Impedance techniques: for the monolayer, bilayer, receptor, 0.5 mg/L estrogen(b) the bilayer, (c) the bilayer with the estrogen receptor, and (d) after exposure of the electrodes to a solution containing 1 ng/mL estrogen.

self assembled decanthiol monolayer on the gold electrode The measurements were performed in the presence of 10 mM $Fe(II)(CN)_6$/$Fe(III)(CN)_6$ as a redox couple. The changes of Z' and Z" of the four layers are clearly shown.

The data present a capacitor C in parallel with a resistor R_p. The values of R and C calculated from the equation $RF_{max}C=1$ (where F is the frequency in radians) are summarized in Table I. As expected for a thicker layer the formation of the bilayer (b) increases R_p and decreases C, compared to the monolayer (a). Penetration of the receptor into the lipid layer further increased the value of R_p and decreased the value of C. In the presence of 0.5mg/L estrogen both R_p and C increased. The results confirm that the impedance technique is an indicative tool to monitor the structural changes in the lipid bilayers on the electrode and, in particular, the effect of the hormone binding to its receptor embedded in the lipid.

AC impedance, as conducted in the latter experiment, was time consuming, especially at low frequencies. The duration of each measurement using single sinusoidal frequency will prevent the use of the technique for online monitoring. Consequently, we considered the possibility of using short pulses; by analyzing the results, one can calculate the equivalent circuit parameters.

TABLE I

Calculated values of R_p and C for the monolayer, bilayer, bilayer-recptor, and bilayer-receptor-estrogen, obtained by AC Impedance measurements..

	Monolayer	Bilayer	Receptor	Hormone
Rp, (Mohm)	.27	.32	.38	.74
C, (microF/cm^2)	3.1	2.65	1.7	1.95

B. Galvanostatic Pulse

Galvanostatic experiments were conducted in phosphate buffer solutions in the absence of a redox couple. Under these conditions, the measurements are more comparable to the natural environment of the hormone and its receptor. We applied short galvanostatic pulses and followed the changes in the potential. The electrodes were left at open circuit potential for 2 min and then a constant current (1 microampere) was applied. Figure 2 depicts the changes in electrode potential during the galvansotatic measurements as obtained for the (a) monolayer, (b) the bilayer, (c) the bilayer after introduction of the receptor and after exposure to 1 pg/mL (0.1 pM) estrogen.

This result can be attributed to an equivalent circuit (shown at the insert of Fig 3). The components of the equivalent circuit were calculated by parameters fitting to Equation (1).

$$E((t)=IRs + Rp1(1-expt/T1) + Rp2(1-exp(t/T2) \qquad (1)$$

where E is the measured potential, I is the applied current, and T is the characteristic life time.

The least square fitting for each curve (4000 data points) was very accurate (least square error in the order of 10^{6}, and the error in each parameter was less than 0.1%). The calculated values of the equivalent circuit components (R_s, $Rp1$, $F?C_1$, $Rp2$, and T_2) for the monolayer, bilayer, bilayer-receptor, and bilayer-receptor-1pg/mL hormone are summarized in Table 2. The capacitances, calculated by $T?Rp_1$, agree with the AC impedance data.

The results shown in Figure 2 indicate that estrogen addition changed the response of the modified electrode. The measured signals showed a rapid increase of the potential followed by a slow stabilization.

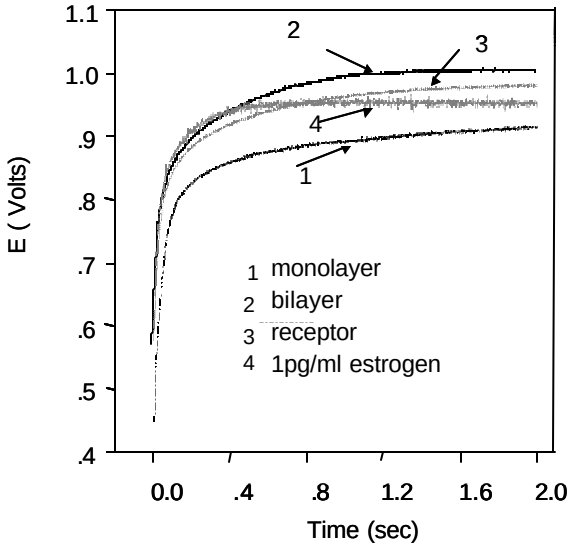

Fig. 2. Changes in electrode potentials during a galvansotatic pulse as obtained for: (1) monolayer, (2) bilayer, (3)embedded receptor, (4)1pg/mL (0.1 pM) estrogen.

TABLE II

Calculated values of the equivalent circuit components: Rs , Rp_1, T_1, $C1Rp_2$ and T_2) for the monolayer, bilayer, bilayer-receptor, and bilayer-receptor-1 pg/mL hormone (n=6). R values are expressed in Mohms T values in seconds and C values are expressed in microfarade/cm^2.

	Monolayer	Bilayer	Receptor	Hormone
Rs,	0.39±0.05	0.53±0.09	0.54±0.06	0.58±0.07
Rp_1,	0.42±0.06	0.29±0.01	0.29±0.06	0.22±0.06
T ??	0.08±0.03	0.04±0.02	0.043±0.01	0.027±0.01
C_1	2.85±0.65	2.26±0.42	2..34±0.27	1..94±0.39
Rp_2	0.14±0.03	0.19±0.04	0.14±0.046	0.16±0.034
T_2	0.89±0.57	0.69±0.48	0.73±0.281	0.22±0.13

Effecst of lower and higher estrogen concentrations are shown in Table III. It should be note that the response of the modified bilayer electrode lacking the receptor was not changed by exposure to 44 pg/mL estrogen. The obtained values of the capacitors and resistors were used to calculate z' and z", and the results are presented in Fig 3.

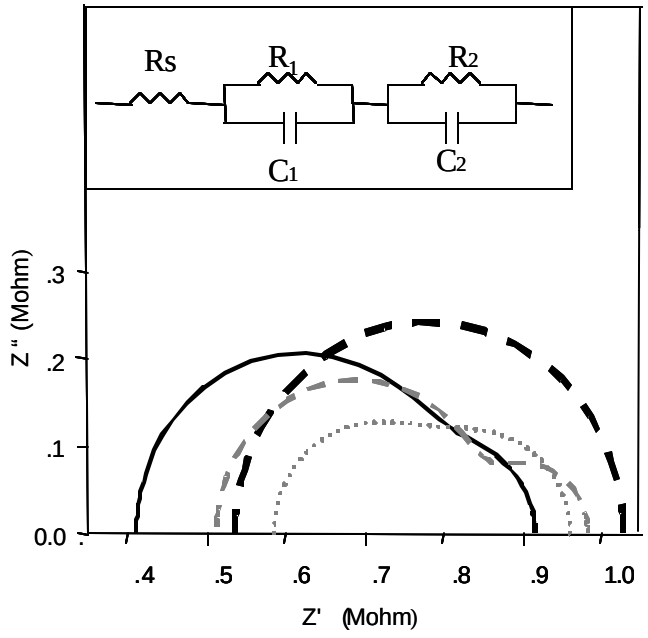

Fig 3 : Nyquist plot for biolayer construction as drawn using the calculated capacitance and resistance values measured by galvanostatic pulses. —: Monolayer, – – –: Bilayer, - - -: Receptor, ·····: 1 pg/ml estrogen. Insert: The equivalent circuit

C. *Xenoestrogen Binding*

Next we examined the potential of the described system to detect interactions with xenoestrogenic. Two xenoestrogens were chosen: one anthropogenic and the other natural. During the last decade, We have applied the sane galvanostatic pulses as with the estrogen. The least squares fitting in (1) yielded the values of R_s, R_{p1}, $T_?$both before and after exposure to estrogen or to xenoestrogens. Bisphenol-A, a plastic softener widely used in industry, was found to be a xenoestrogen that binds to the estrogen receptor. The effects of estrogen, bisphenol-A, and genistein on the electrical properties of the bilayer-receptor-modified electrode are summarized in TABLE III. Cross-reactivity studies using an estrogen receptor biological assay> @have shown that receptor is activated 1000 times less by bisphenol-A than by estrogen. The effect of Bisphenol-A was apparent at the ng/mL range. Similar to the results obtained with estrogen, higher concentrations showed only a small change. Estrogen-receptor activation by the phytoestrogen genistein is 30 times less than by estrogen but 30 times more than by bisphenol-A. The change in the electrode properties before and after exposure the embedded receptor to 1 ng/mL genistein. is shown in TABLE III, The electrochemical results agree with those of the cross-reactivity bioassay in the literature.

Table III shows that the bilayer-receptor-modified electrode gave a similar response to either the natural hormone or xenoestrogen. The R_s values increased, R_{p1} values decreased, the lifetime $T_?$ values decreased, and accordingly the calculated C_1 values decreased. These alterations can be attributed to the dimerization and conformational changes of the receptor, which increase its hydrophobicity and allow it to enter into the lipid layer. Currently, we are conducting atomic force microscopy and quartz crystal micro-balance experiments to validate our finding. Preliminary results support the described model.

TABLE III
Summary of the effects of exposure to differet estrogen and xenoestrogen concentrations on the electrical properties of the bilayer-receptor modified electrodes. Numbers denote % of change, NC – no change.

	Rs	T	Rp	C
0.015 pg/ml Estrogen	NC	NC	NC	NC
44pg/ml Estrogen	74.28	-19.96	-45.74	-28.98
30ng/ml Bisphenol-A	24.43	-56.63	-16.4	-47.34
3000g/ml Bisphenol-A	33.84	-63.26	-26.54	-48.69
1 ng/ml Genistein	56.28	-21.71	-30.18	-9.78

IV CONCLUSIONS

This paper presents a follow-up of binding events on a lipid bilayer-modified electrode after exposure to either estrogen or xenoestrogens. The supported lipid layers mimic a natural receptor biomembrane environment, allowing ligand binding that leads to conformational changes in the bilayer. Using fast impedance techniques to reveal the electrical properties of the biolayer before and after binding, we could detect estrogen or xenoestrogens effects in the range of picograms per milliliter solution. The responses to the xenoestrogens Bisphenol-A and genistein correlate with those of traditional biological assays reported in the literature. Such a system might be a key to identifying and defining chemicals or pollutants as potent xenoestrogens *in-situ* and lead to a highly sensitive monitoring method by eliciting an immediate response to a wide range of xenoestrogens, either known or unknown.

At present we are examining the use of disposable screen-printed electrodes (gold as a working electrode), and the results are comparable to those described above, providing a low cost, and easy to use sensor that can be used routinely for continuous control of endocrine disrupters in industry . areas or in other suspect locations. The technique presented here can also serve as a platform for the study of universal receptor hormone interactions.

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