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Impedance Biosensors: Applications to Sustainability and Remaining Technical Challenges

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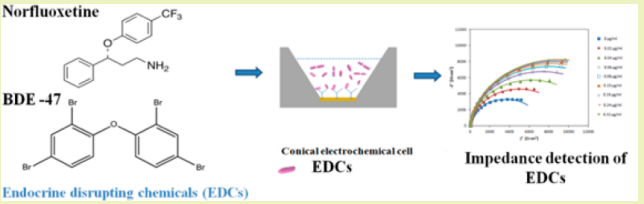
ABSTRACT: Due to their all-electrical nature, impedance biosensors have significant potential for use as simple and portable sensors for environmental studies and environmental monitoring. Detection of two endocrine-disrupting chemicals (EDC), norfluoxetine and BDE-47, is reported here by impedance biosensing, with a detection limit of 8.5 and 1.3 ng/mL for norfluoxetine and BDE-47, respectively. Although impedance biosensors have been widely studied in the academic literature, commercial applications have been hindered by several technical limitations, including possible limitations to small analytes, the complexity of impedance detection, susceptibility to nonspecific adsorption, and stability of biomolecule immobilization. Recent research into methods to overcome these obstacles is briefly reviewed. New results demonstrating antibody regeneration atop degenerate (highly doped) Si are also reported. Using 0.2 M KSCN and 10 mM HF for antibody regeneration, peanut protein Ara h 1 is detected daily during a 30 day trial.



KEYWORDS: *Electrochemical impedance spectroscopy, Biosensors, Sustainability, Endocrine-disrupting chemicals, Nanotechnology*

INTRODUCTION

Biosensors that utilize electrochemical impedance spectroscopy for signal transduction have been widely studied in the academic literature, with several authoritative reviews.[1−3] Impedance biosensing involves application of a small amplitude AC voltage to the sensor electrode and measurement of the in/out-of-phase current response as a function of frequency. Impedance biosensors are fabricated by immobilizing a biorecognition molecule onto a conductive and biocompatible electrode and then detecting the change in the interfacial impedance upon analyte binding.[1−3] Biorecognition molecules may include antibodies, receptor proteins, single-stranded DNA, aptamer, or peptides. Although impedance biosensors have been well studied in the academic literature,[1−3] commercial applications have not ensued, so technical challenges to usage of impedance biosensors are reviewed here.

For small molecule analysis, the actual structure targeted is approximately the size of the molecule that can be recognized by an antibody. Although small molecules (<1000 Da) alone cannot induce an immune response, they can be antigenic or recognized by an antibody.[4] To render a small molecule immunogenic, it must be conjugated to a larger carrier molecule (i.e., a protein) via a functional group.[5] The simplest and most convenient source of antibodies is the sera of an immunized animal (usually a mouse, rabbit, sheep, or goat). This sera contains a heterogeneous mixture of antibodies of varying affinity, termed polyclonal antibodies. Polyclonal antibodies can be employed as this mixture, or they can be further purified using standard techniques, such as immunoaffinity chromatography.[6]

Due to its all-electrical nature, impedance biosensors are simpler than other methods because they lack optical or acoustic components, offering significant advantages for portable applications.[7] This makes impedance biosensors ideal for environmental monitoring of species such as endocrine-disrupting chemicals (EDCs),[8] diarrhetic shellfish poisoning (DSP) toxins,[9,10] polychlorinated biphenyls,[11] and milk toxins such as veterinary drug residues and hormones.[12,13] New results are presented here for impedance detection of norfluoxetine and BDE-47, demonstrating that even relatively small organic molecules can be detected by this method. Technical challenges that have hindered commercialization of impedance biosensors are briefly reviewed, including possible limitations to small analytes, the complexity of impedance detection, susceptibility to nonspecific adsorption, and stability of biomolecule immobilization. New results are also presented demonstrating antibody regeneration atop degenerate (highly doped) Si for a 30 day trial using 0.2 M KSCN and 10 mM HF in the denaturing solution.

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EXPERIMENTAL METHOD

Electrode and Biosensor Preparation. Glass slides with a 100 nm Au film atop a 5 nm Ti adhesion layer were purchased from Evaporated Metal Films (Ithaca, NY). As-doped (n-type) degenerate silicon (111) wafers with a thickness of 500 μm and a diameter of 50 mm were purchased from University Wafers. 11-Mercaptoundecanoic acid (11-MUA) and 10 undecanoic acid were purchased from Aldrich. N-(3-(Dimethylamino)propyl)-N′-(ethylcarbodiimide hydrochloride) (EDC), potassium dihydrogen phosphate, and dipotassium dihydrogen phosphate were purchased from Sigma. *N*-hydroxysulfosuccinimide sodium salt (NHSS) was purchased from Pierce biotechnology, and potassium ferri/ferrocyanide was purchased from Acros Organics. Potassium thiocyante was purchased from Fisher Scientific. HF dip was purchased from J.T. Baker. Bovine serum albumin was purchased from Jackson Immnuo Research Laboratories. Peanut protein Arah 1 and its monoclonal antibody were purchased from Indoor Biotechnologies. Norfluoxetine was purchased from Santa Cruz Biotechnology, and its sheep polyclonal antibody was purchased from Abbiotec. BDE-47 was purchased from Chem Services, and rabbit polyclonal antibodies raised to BDE-47 were protein A purified and stored at 4 mg/mL in a phosphate-buffered saline.[14]

The n-type degenerate Si(111) electrode was embedded within a virgin Teflon mount with an electrode area of 0.19 cm^2 and a cell volume of 1 mL, cleaned in ethanol and water, and etched in 10:1 HF dip to remove the native oxide. This was immediately immersed into 10% 10-undecanoic acid in deaerated toluene for 19 h, with exposure to 352 nm ultraviolet light for photoactivated alkene insertion into Si−H bonds, creating a carboxylate-terminated surface. The Au electrode was fixed by an O-ring onto an electrochemical cell constructed from virgin Teflon with an electrode area of 0.19 cm^2 and a cell volume of 6 mL. The Au electrode was cleaned with ethanol, dried, and immersed for 17 h into 1 mM 11-MUA and 50 mM phosphate buffer solution (pH 10) to form a carboxylate-terminated self-assembled monolayer (SAM). The carboxylate groups on both Au and Si electrodes were then activated for 1 h in 75 mM EDC and 15 mM NHSS in 50 mM phosphate buffer solution (pH 7.3). The antibody-coated electrodes were created by immersion for 1 h into a solution containing 50 μg/mL antibody and 50 mM PBS at pH 7.3, forming amide bonds to amine groups on the protein surface, and then immersed into 0.1% BSA for 1 h to reduce the nonspecific adsorption.

Experimental Methods. All electrochemical measurements were performed with a three-electrode configuration using a Pt spiral counter electrode and a Ag/AgCl reference electrode. The background test solution contained 50 mM PBS and 5 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ at pH 7.4, with varying concentrations of the target analyte. Impedance measurements were performed using a Gamry instruments Reference 600 over the frequency range from 0.01 Hz to 15 kHz with an AC probe 120 amplitude of 5 mV. Each impedance spectrum takes about 2.8 min to acquire. The impedance results were obtained at a DC potential of +200 mV vs Ag/AgCl for the gold electrode and at a DC potential that is slightly cathodic to the open circuit potential (OCP) vs Ag/AgCl for Si electrode. In some systems, the impedance response may be unstable if the applied DC potential is too far from the open circuit potential.[15]

RESULTS AND DISCUSSION

Technical Challenges for Impedance Biosensors. Impedance biosensors are widely considered to have some technical limitations that have hindered their commercial introduction, including (1) possible limitations for detection of small analytes, (2) difficulty of simplifying and miniaturizing AC impedance detection, (3) susceptibility to nonspecific adsorption in complex matrices, and (4) stability and reproducibility for biomolecule immobilization onto a conductive electrode material.

Many of these challenges are not specific to impedance biosensors. For example, nonspecific adsorption is a common problem for all biosensor transduction methods.

Impedance Detection of Small Analytes. Endocrine-disrupting compounds (EDCs) are an emerging class of contaminants that can disrupt the endocrine system in animals, resulting in a range of possible health problems, including birth defects and other developmental disorders.[8] This has resulted in widespread concern about the presence of EDCs in the environment, particularly in water, wastewater, and agricultural irrigation. However, specific environmental regulations are still unclear, in part due to the large number of EDCs and the lack of detailed understanding of their source, distribution, and physiological effects.[16−19] Simple portable biosensors that utilize impedance methods would be invaluable for understanding the source and distribution of EDCs, which are typically organic molecules of low molecular weight.

The detection limits reported for impedance biosensors in the academic literature generally range from 10^{-15} to 10^{-6} M.[1−3] Impedance detection of small molecules is expected to be difficult due to the exponential increase of the charge transfer resistance (R_{ct}) with tunneling distance (x) through the polymer−protein film to the underlying electrode[20]

$$R_{ct} = R_{ct}^{0} e^{\beta x} \tag{1}$$

where R_{ct}^{0} and β are constants, with β typically in the range of 0.05−0.11/nm at Au−thiol interfaces.[20] Thus, larger changes in x associated with binding of larger analytes are more easily detected. The fundamental underpinning for eq 1 is the exponential decline in the electron transfer rate (k_{ET}) with distance from the electrode surface at distances greater than atomic dimensions (~0.3 nm)[21]

$$k_{ET} = 10^{13} e^{-\alpha(x-0.3)} e^{-(\Delta G^{\circ} + \lambda)^2/4RT\lambda} \tag{2}$$

where α is a constant for a given redox couple, and λ is the Marcus reorganization energy. The charge transfer resistance (R_{ct}) is inversely related to the rate of electron transfer (k_{ET}) through the polymer−protein film. Impedance detection of endocrine-disrupting chemicals has been previously reported by several groups,[22−25] and two additional examples are given below for purposes of illustration.

Norfluoxetine is an active metabolite of fluoxetine and has been investigated by Eli Lilly as an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class, with a molecular weight of only 295.30. Figure 1 is a Nyquist plot illustrating impedance detection of norfluoxetine at an Au electrode onto which its sheep polyclonal antibody is immobilized.

The results from Figure 1 are fit to the Randles equivalent circuit shown in Figure 2 but with the differential capacitance (C_d) replaced by a constant phase element (CPE) whose impedance (Z) is[26−28]

$$Z(\mathrm{CPE}) = \frac{1}{T(j\omega)^n} \tag{3}$$

The constant phase element can be viewed as a heuristic method to incorporate the effects of surface heterogeneity both along and through the electrochemical interface.[26−28] The other circuit elements in Figure 2 are the charge transfer resistance (R_{ct}) and solution resistance (R_S). Although other applications of electrochemical impedance spectroscopy may involve complex equivalent circuits, the Randles equivalent circuit is almost always employed for impedance biosensors. The best-fit equivalent circuit parameters are given in Table 1 as a function of norfluoxetine concentration over the frequency

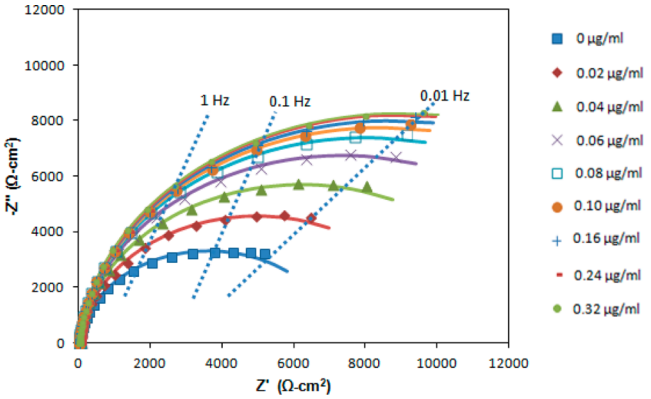

Figure 1. Nyquist plot of the interfacial impedance of the antibody-coated electrode after exposure to norfluoxetine.

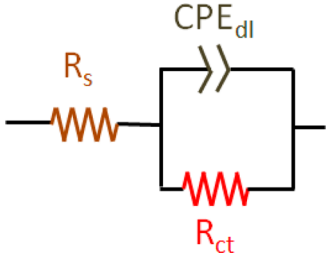

Figure 2. Randles equivalent circuit, with the differential capacitance (C_d) replaced with the constant phase element (CPE).

range of 0.05 Hz to 15 kHz. Figure 3 illustrates the increase in the charge transfer resistance (R_{ct}) with increasing concentration of norfluoxetine. R_{ct} approaches a constant value at high norfluoxetine concentration as the antibody film gradually becomes saturated. R_{ct} is typically the most sensitive of the equivalent circuit elements in Table 1 to analyte binding. Thus, many frequencies are insensitive to analyte binding, so impedance biosensors can be operated at only one or a few frequencies that are most sensitive to analyte binding.[15] This also reduces the detection time, in some cases allowing for real time impedance biosensing.[15]

On the basis of the determination of R_{ct}, the linear range extends approximately to 0.07 μg/mL, and the detection limit for norfluoxetine is approximately 8.5 ng/mL or 28 nM. This is comparable to the norfluoxetine detection limits reported from high pressure liquid chromatography (10 ng/mL) and gas chromatography (2 ng/mL) measurements.[29,30] This is also well below physiological levels (72−258 ng/mL) reported by the U.S. Food and Drug Administration (FDA) following ingestion of fluoxetine hydrochloride.[31]

2,2′,4,4′-Tetrabromodiphenyl ether (BDE-47), with a molecular weight of 485.6, provides another demonstration of impedance detection of small organic analytes. Increasing concentrations of polybrominated diphenyl ethers (PBDEs) in

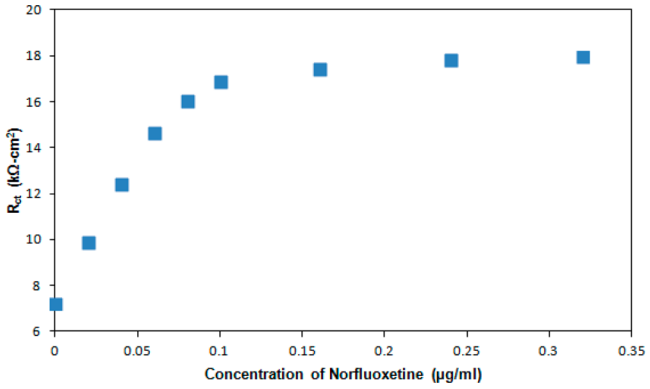

Figure 3. Variation of charge transfer resistance (R_{ct}) with norfluoxetine concentration.

the environment, human food chain, and human tissues raise concern about possible neurotoxic effects.[32] PBDEs are used as flame retardants in a range of products, such as electronic equipment, furniture, construction materials, and textiles. In most cases, BDE-47 is the predominant congener. Accumulation of BDE-47 is more rapid in infants than adults due to their diet (breast feeding/relatively large intake) and contact with house dust.[33] Behavioral studies have demonstrated adverse neurodevelopmental effects on learning and memory after BDE-47 exposure at the critical stage of neonatal brain development.[34] As a demonstration, Figure 4 presents the

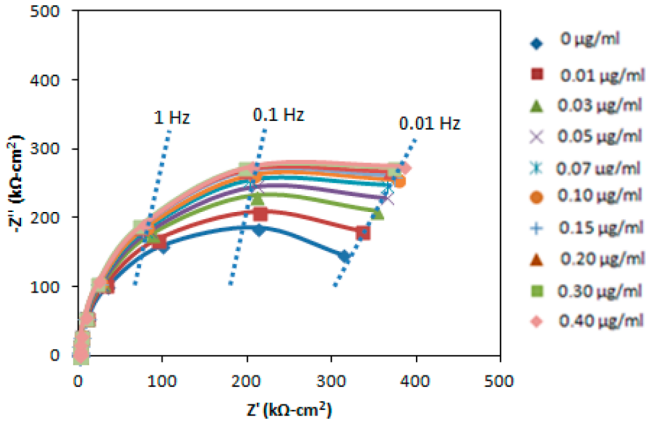

Figure 4. Nyquist plot of the interfacial impedance of the antibody-coated electrode after exposure to BDE-147.

Nyquist plot for the impedance detection of BDE-47 at a Au electrode on which its rabbit polyclonal antibody is immobilized. The best-fit equivalent circuit parameters are given in Table 2 as a function of BDE-47 concentration. Similar to norfluoxetine, the detection limit of BDE-47 was estimated

Table 1. Best-Fit Equivalent Circuit Parameters (Standard Errors) with Increasing Norfluoxetine Concentration

norfluoxetine concentration (μg/mL)	0	0.02	0.04	0.06	0.08	0.1	0.16	0.24	0.32
R_s (Ω cm^2)	19.4 (0.2)	19.9 (0.2)	21.6 (0.1)	21.9 (0.1)	21.8 (0.2)	21.8 (0.1)	21.9 (0.1)	22.5 (0.1)	23.3 (0.2)
CPE-T (μF cm^{-2} s^{n-1})	2.27 (0.01)	2.25 (0.01)	2.23 (0.01)	2.22 (0.01)	2.21(0.01)	2.20 (0.01)	2.20 (0.01)	2.20 (0.01)	2.20 (0.01)
n	0.95 (0.01)	0.95 (0.01)	0.95 (0.01)	0.95 (0.01)	0.95 (0.01)	0.95 (0.01)	0.95 (0.01)	0.95 (0.01)	0.95 (0.01)
R_{ct} (kΩ cm^2)	7.21 (0.14)	9.91 (0.15)	12.4 (0.2)	14.7 (0.2)	16.1 (0.2)	16.9 (0.2)	17.4 (0.2)	17.8 (0.2)	18.0 (0.2)

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Table 2. Best-Fit Equivalent Circuit Parameters (Standard Errors) Following Exposure to Different BDE-47 Concentrations

BDE-47 concentration (μg/mL)	0	0.01	0.03	0.05	0.07	0.1	0.15	0.2	0.3	0.4
R_s (Ω cm^2)	57.07 (0.6)	56.38 (0.6)	56.32 (0.6)	55.99 (0.6)	55.59 (0.5)	56.73 (0.6)	56.67 (0.5)	55.92 (0.6)	56.67 (0.6)	57.16 (0.6)
CPE-T (μF cm^{-2} s^{n-1})	3.97 (0.04)	3.97 (0.04)	3.98 (0.04)	3.97 (0.04)	4.02 (0.04)	3.97 (0.04)	4.01 (0.04)	4.05 (0.04)	4.03 (0.04)	3.96 (0.04)
n	0.96 (0.01)	0.96 (0.01)	0.95 (0.01)	0.96 (0.01)	0.96 (0.01)	0.96 (0.01)	0.95 (0.01)	0.96 (0.01)	0.96 (0.01)	0.96 (0.01)
R_{ct} (kΩ cm^2)	396 (6)	449 (8)	501 (6)	537 (6)	568 (7)	586 (7)	602 (7)	613 (6)	621 (7)	625 (7)

as 1.3 ng/mL (2.7 nM) from the measurement of R_{ct} and sensitivity.

If impedance detection of low molecular weight EDCs proves challenging, then improved sensitivity can be attained in a direct impedance immunoassay format by analyte conjugation with electrochemically bright metal and semiconductor nanomaterials, particularly Au nanoparticles.[35−37] In addition, enhanced sensitivity can be achieved with alternative formats such as an impedance displacement assay.[38,39]

The effect of BDE-47 binding at the biosensor interface can also be visualized by cyclic voltammetry of the antibody-coated electrode, as illustrated in Figure 5. As the BDE-47 concentration is increased, the increasing surface coverage of BDE-47 progressively blocks charge transfer to/from $[Fe(CN)_6]^{3-/4-}$, resulting in lower reduction/oxidation peak heights in Figure 5.

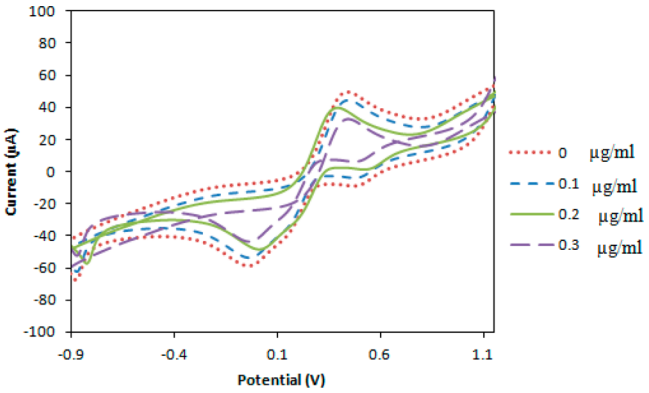

Figure 5. Cyclic voltammograms of antibody-coated electrode exposed to increasing concentrations of BDE-47.

Complexity of Impedance Detection Electronics. Another frequent concern with impedance biosensors is the more complex measurement circuitry relative to amperometry, which has already been miniaturized and commercialized for transdermal glucose biosensors.[40,41] However, the research group of Dr. Suh-Moon Park has published extensively on Fourier transform electrochemical impedance spectroscopy (FTEIS) for a wide variety of electroanalytical applications, including electrocatalysis and corrosion.[42,43] During FTEIS, impedance spectra are obtained by a relatively simple procedure that involves application of a potential step function, measurement of the current response at the electrochemical interface, differentiation, and fast Fourier transformation (FFT) back into frequency space.[42,43] The first reports of FFT analyses of electrochemical systems by this type of probe−response approach actually date to Dr. Donald Smith in 1976.[44,45] This approach has been criticized as being unable to obtain high frequency spectra due to limitations on sampling frequency and unable to obtain low frequency spectra due to limitations on experimental duration.[46] However, inspection of Figures 1 and 4 reveals that information about analyte binding is contained mainly at intermediate frequencies, so these limitations do not significantly impact the utility of this method for impedance biosensing.

More recently, hardware simplifications for FTEIS have been reported to reduce the cost of biosensor applications.[47] For situations where an electrochemical system is not at steady state, then dynamic FFTEIS may allow a more rapid snapshot of an electrochemical system.[48,49] Impedance methods for biosensing can also be combined with other transduction methods, such as surface plasmon resonance biosensing.[50,51]

Susceptibility to Nonspecific Adsorption. The most frequently cited practical concern regarding impedance biosensors is the perception that this method is particularly susceptible to interference arising from nonspecific adsorption. Nonspecific adsorption is without question a common limitation for a wide variety of different biosensor methodologies.[52−54] Nonspecific adsorption is typically ascribed to proteins contained in a complex test matrix binding to the sensor interface though an unwanted process not involving biomolecular recognition. Thus, nonspecific adsorption can be studied by control experiments using either complex test matrices or mixtures of different proteins or analytes.

While nonspecific adsorption may cause spurious signals during impedance biosensing, several methods have been employed to mitigate this, including sample dilution,[55,56] adsorption of a blocking reagent such as bovine serum albumin (BSA),[54] and use of a control electrode at which biomolecular recognition is unlikely.[57,58] The utility of sample dilution depends on the particular application and the desired detection limit. When a monoclonal antibody is used for biomolecular recognition, a control electrode can be used with another antibody from the same animal and subtype whose antigen is unlikely to be found in the text matrix of interest. Recently, we reported impedance detection of *Listeria monocytogenes* in tomato pulp[58] and demonstrated that nonspecific adsorption was unmeasurable. Nonspecific adsorption was quantified by comparing the impedance change at the measurement electrode (mouse monoclonal IgG_1 antibody to *L. monocytogenes*) to that at a control electrode (mouse monoclonal IgG_1 antibody to GAPDH). This approach depends on the availability of adequate control electrodes whose antigen is not present in the samples of interest and with no cross-reactivity to the analyte of interest. It should be noted that the use of multiple measurement antibodies with different binding epitopes and multiple control antibodies are both relatively straightforward.

Impedance detection of norfluoxetine and BDE-47, which is illustrated in Figures 1 and 4, respectively, has not yet been demonstrated in complex media. However, nonspecific adsorption is sometimes assessed by simply exposing the

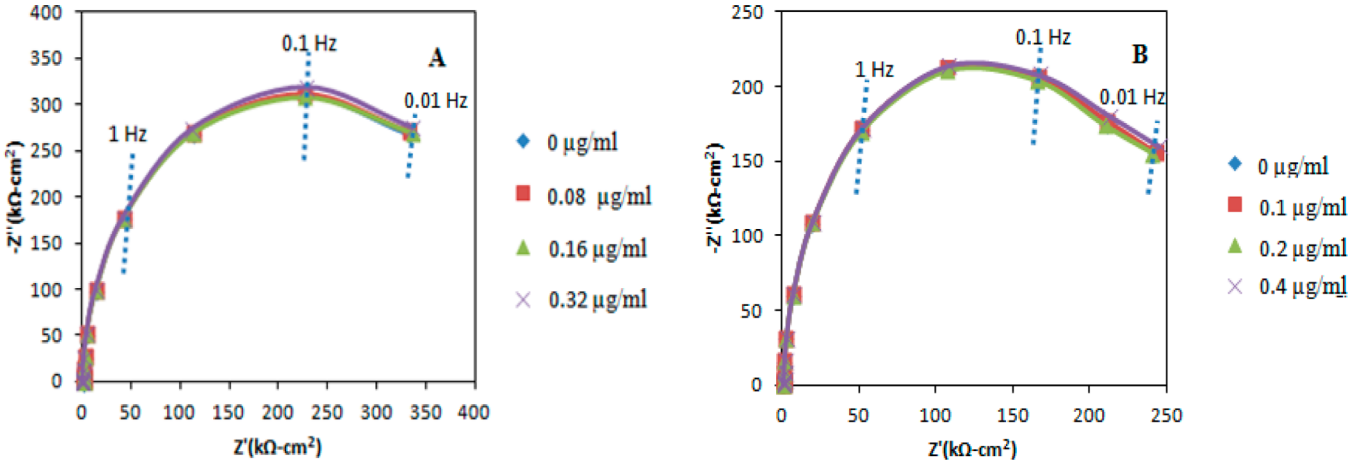

Figure 6. Nyquist plot of the impedance response of (A) the BDE-47 antibody-coated coated electrode after exposure to the Norfluoxetine, and (B) Norfluoxetine antibody-coated electrode after exposure to the BDE-47.

antibody-coated electrode to solutions of known proteins or to analyte mixtures. Electrodes at which the antibodies to norfluoxetine and BDE-47 have been immobilized were exposed to norfluoxetine, BDE-47, peanut protein Ara h 1, and *Cyprinus carpio* vitellogenin. In all cases, impedance changes were observed only for the analyte of interest, and none of the interfering species caused a change in the impedance spectrum. This is illustrated by the results of Figures 6A and B, where increasing concentrations of norfluoxetine (BDE-47) do not cause any interference at the BDE-47 (norfluoxetine) antibody-coated electrode, demonstrating both that nonspecific adsorption does not occur and that these antibodies are not cross-reactive. Similar results are obtained upon exposure of these electrodes to peanut protein Ara h 1 and *Cyprinus carpio* vitellogenin, demonstrating that nonspecific adsorption does not occur in this system, at least for these proteins. For low molecular weight analytes such as endocrine-disrupting chemicals (EDCs), antibody cross-reactivity may sometimes be observed.[59,60]

Stability of Biomolecule Immobilization onto a Conductive Electrode Material. For impedance biosensing, biomolecule immobilization onto a conductive and biocompatible electrode material is most commonly accomplished through Au−thiol self-assembly chemistry.[61] However, the limited stability of Au−thiol self-assembly chemistry to date limits its application to impedance biosensors.[62] Depending on storage conditions, the shelf life is limited to days to weeks. Durable chemistry for biomolecule immobilization is also needed for sensor calibration, which often involves the use of aggressive chemicals for antibody denaturation.

Other substrate materials that have been reported for impedance biosensors include carbon,[63,64] Si,[65,66] Pt,[67,68] Ti,[69,70] and ITO.[71,72] Recently, degenerate (highly doped) Si was reported as an alternative electrode material for impedance biosensors.[73] Degenerate Si behaves as an electrical conductor, albeit a poor one, rather than a semicondutor, preventing formation of a space charge layer during AC interrogation of the sensor interface. Figure 7 illustrates new results demonstrating the ability to regenerate a Si electrode during a 30 day trial period.[74] During these experiments, the antibody-coated Si electrode was stored in 50 mM PBS buffer at pH 7.3. This was removed and tested every day for 30 days using the following procedure. The electrode was exposed to peanut protein Ara h 1 at a concentration of 0.04 μg/mL, exposed to

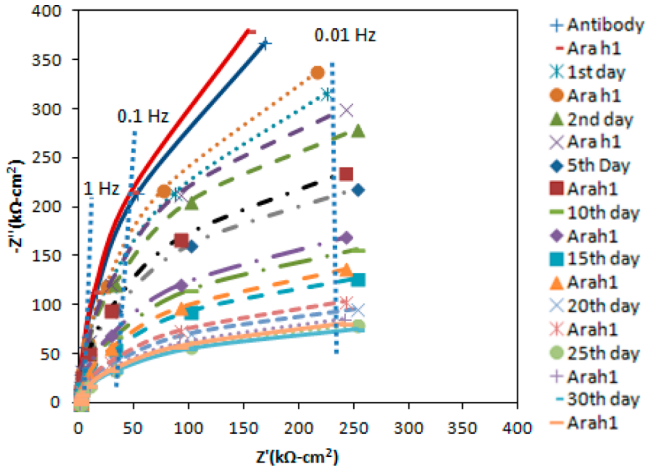

Figure 7. Nyquist plot of the interfacial impedance for the regeneration of Si electrode for 30 days. Test solution contains 50 mM PBS and 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ at pH 7.3.

0.2 M KSCN and 10 mM HF to unfold the antibody film and release the analyte, and exposed to 0.1 M BSA and 50 mM PBS buffer to refold the antibody film. The inclusion of HF in the unfolding solution is necessary to dissolve Si oxide that forms during electrode storage. In order to avoid congestion, Figure 7 only illustrates the data taken every fifth day. While the response of the antibody film degrades, degraded, the response within any 1 day is consistent to within 2%. This illustrates the potential for this methodology to be used for storage of antibody-coated degenerate Si electrodes, with calibration on the day they are used. Although the results of Figure 7 have not been demonstrated for endocrine-disrupting chemicals (EDCs), the physical chemistry of different antibodies is quite similar, so this chaotropic agent (0.2 M KSCN and 10 mM HF) should work on other antibodies as well.

CONCLUSIONS

Detection of two endocrine-disrupting chemicals (EDC), norfluoxetine and BDE-47, is reported here by impedance biosensing, with a detection limit of 8.5 and 1.3 ng/mL for norfluoxetine and BDE-47, respectively. Recent research into possible limitations of impedance biosensors are briefly reviewed, including possible limitations to small analytes, the

complexity of impedance detection, susceptibility to nonspecific adsorption, and stability of biomolecule immobilization. New results demonstrating antibody regeneration atop degenerate (highly doped) Si are also reported. Using 0.2 M KSCN and 10 mM HF for antibody regeneration, peanut protein Ara h 1 is detected daily during a 30 day trial.

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Notes

The authors declare no competing financial interest.

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