Quantifying Hormone Disruptors with an Engineered Bacterial Biosensor

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ABSTRACT: Endocrine disrupting compounds are found in increasing amounts in our environment, originating from pesticides, plasticizers, and pharmaceuticals, among other sources. Although the full impact of these compounds is still under study, they have already been implicated in diseases such as obesity, diabetes, and cancer. The list of chemicals that disrupt normal hormone function is growing at an alarming rate, making it crucially important to find sources of contamination and identify new compounds that display this ability. However, there is currently no broad-spectrum, rapid test for these compounds, as they are difficult to monitor because of their high potency and chemical dissimilarity. To address this, we have developed a new detection strategy for endocrine disrupting compounds that is both fast and portable, and it requires no specialized skills to perform. This system is based on a native estrogen receptor construct expressed on the surface of Escherichia coli, which enables both the detection of many detrimental compounds and signal amplification from impedance measurements due to the binding of bacteria to a modified electrode. With this approach, sub-ppb levels of estradiol and ppm levels of bisphenol A are detected in complex solutions. Rather than responding to individual components, this system reports the total estrogenic activity of a sample using the most relevant biological receptor. As an applied example, estrogenic chemicals released from a plastic baby bottle following microwave heating were detectable with this technique. This approach should be broadly applicable to the detection of chemically diverse classes of compounds that bind to a single receptor.

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are increasingly identified as potent and pervasive risks to human health. They enter the environment through numerous human activities, including pesticide use, agriculture, and fracking, and they are found in consumer products such as plastic kitchen products and food can linings.1–3 EDCs are especially dangerous because they are harmful at very low concentrations (picomolar to nanomolar), particularly to fetuses and newborns,4–8 and they are implicated in increased occurrences of obesity, diabetes, infertility, and cancer.9–11 The rapid and sensitive detection of these chemicals is therefore vital, ideally using equipment that is portable and inexpensive. Unfortunately, these compounds are particularly difficult to measure because they are not defined by a common chemical structure, but instead by their activity.12,13 To address this obstacle, we have developed a new detection paradigm for the sensitive, broad-spectrum detection of EDCs based on a native estrogen receptor alpha (ERα) construct expressed on the surface of Escherichia coli. These engineered bacterial sensors enable the detection of many detrimental compounds as well as signal amplification from impedance measurements as they bind to modified electrodes. Rather than responding to individual components, this approach reports the total estrogenic activity of a sample using the biological receptor itself. Additional features of this sensing strategy include sample volumes of only 10 μL, rapid response rates, and the use of low-cost, disposable electrodes. As such, it is the first broad-spectrum EDC assay that is appropriate for field use.

The current standards for EDC detection are cell-based assays (originally the E-SCREEN assay14 and, more recently, transactivation assays15,16 and yeast-based assays17,18) and radioactive19 and fluorescent competition assays.20,21 The cell-based transactivation involves the transcription of a reporter gene, such as a luciferase gene, following the addition of the compound in question. While effective, this analytical method is problematic for rapid, point-of-care application, as it can require multiple days of cell culture, specialized equipment, and trained laboratory personnel. Similar problems arise with fluorescent polarization assays, in which fluorescently labeled 17β-estradiol is displaced from specific antibodies by estrogenic compounds. This method requires several conjugation reactions and optimization steps, and a specialized fluorometer is necessary for measurement. As alternatives, efforts have been made to develop rapid EDC detectors, including both fluorescent and electrochemical sensors.2,22 While these platforms have had success in detecting specific compounds or chemical families, most are based on the binding of a single type of small molecule to a particular antibody or DNA aptamer, precluding
broad detection of estrogenic activity (EA). Furthermore, antibodies can introduce cost and storage difficulties, and many platforms require analyte labeling with an electrochemical probe or fluorophore for detection.

The approach described herein is based on a novel electrochemical sandwich assay (Figure 1a) and involves the use of lyophilized *E. coli* to cause changes in the surface impedance upon binding. Several unique aspects of this strategy enable the detection of a range of estrogenic compounds at exceptionally low concentrations. The *E. coli* surfaces are engineered to display the ERα capture agent, which facilitates detection of any compounds that associate with its binding pocket. The use of lyophilized *E. coli* limits their viability and increases storage life. The second component of the sandwich assay is an electrochemical working electrode modified with a previously reported protein that binds to ERα only when a ligand is present. This protein is attached through the interactions of a cysteine thiol with a disposable gold electrode surface (Figure 1b). The specificity of the monobody is observable by scanning electron microscopy of the working electrode surfaces. In the presence of estradiol (E2), *E. coli* was observed on the surface, while in the absence of E2, no *E. coli* bound the surface (Figure 1c,d).

**RESULTS AND DISCUSSION**

The use of lyophilized *E. coli* as a scaffold for the ERα protein resulted in significantly more sensitive measurements of E2 compared to the binding of ERα alone (Figure 2). The
enhanced sensitivity is due to a substantially increased impedance response from the recruitment of the large E. coli cells to the gold surface, as compared to the significantly smaller free protein (Figure 2b). Additionally, no signal change is observed in the presence of E2 but with no E. coli or ERα added (Figure S3). Both fresh and lyophilized E. coli were tested, and a dependence on the number of cells used for detection was observed (Figure S1). For lyophilized cells, the optimal number of cells was found to be 10^7/mL. The number of ERα proteins surface expressed on fresh E. coli was determined to be approximately 70,000 using a fluorescent coumarin−E2 conjugate, while on the lyophilized E. coli it was slightly lower (50,000/cell) (Figure S2). This level of surface expression is expected, as the maximum number of ice
nucleation proteins that were fused to ERα is on the order of 100,000.\textsuperscript{27,28} Fresh \textit{E. coli} resulted in a small impedance response as compared to the lyophilized cells (Figure 2b) likely due to their motility, which reduces their binding to the electrode surface. This hypothesis was supported by comparing detection with \textit{E. coli} killed with sodium azide to \textit{E. coli} rendered nonviable, but alive and motile, by a low dose of chloramphenicol. The chloramphenicol-treated \textit{E. coli} behaved as the untreated, live \textit{E. coli}, and the sodium azide treated cells behaved similarly to the lyophilized cells (Figure S3). Consistent with this behavior, no \textit{E. coli} from a live sample were observed on electrodes by electron microscopy.

Detection of the binding event was accomplished with electrochemical impedance spectroscopy (EIS) in ferricyanide/ferrocyanide solution. This technique is rapid (providing readout in minutes), sensitive, and label-free.\textsuperscript{29,30} Nyquist plots were generated from each EIS scan performed, and the data were fit to a constant phase element (CPE) circuit model (Figure 2a). The charge transfer resistance (\(R_{\text{CT}}\)) was derived from the CPE fits and was found to be proportional to the amount of ERα bound to the electrode and, therefore, the amount of substrate present. Using \(R_{\text{CT}}\) as a proxy for the concentration of substrate, we were able to detect 500 pM E2 with a large linear range of detection up to 10 \(\mu\text{M}\) (Figure 2b).

As the required sample volume is especially low (10 \(\mu\text{L}\)), we were able to detect femtomoles of estradiol at the detection limit.

The system was found to be especially versatile, with detection of chemicals that have disparate chemical structures but similar bioactivity. The EDCs tested that bind ERα are 4-nonylphenol (4-NP), genistein (GEN), diethylstilbestrol (DES), and bisphenol A (BPA). Progesterone (P4) was used as a negative control, as P4 is not a substrate for ERα (DES), and bisphenol A (BPA). Progesterone (P4) was used as a negative control, as P4 is not a substrate for ERα.

The gene encoding for the ERα vector. The gene encoding for the ERα monobody protein, a sequence adapted from Koide et al.,\textsuperscript{19,20} Plasmid Preparation. \textit{Monobody Encoding pSKB3 Vector}. The gene encoding for the ERα monobody protein, a sequence adapted from Koide et al.,\textsuperscript{19,20} was synthesized by IDT Technologies with \textit{Bam}HI and \textit{Xho}I restriction sites at the 5’- and 3’-ends and subcloned into a pSKB3 vector—a variation of Novagen’s pET-28a vector with the thrombin site exchanged for a TEV proteolysis site. The

Independent of the ratio of EDCs in the solution, the \(R_{\text{CT}}\) was found to be comparable to the equivalent concentration of E2 (Figure 4b). This platform therefore shows the distinct advantage of providing a readout of the total EA from a complex mixture of components even when their specific identities are unknown.

Importantly, this approach enables detection of target compounds present in complex mixtures of proteins and small molecules. As EDCs are especially deleterious for proper development, their presence has been especially problematic in infant products. As one relevant example, the detection of BPA was evaluated in infant formula (Figure 4a). BPA was added to reconstituted formula from a commercial source in varying concentrations. The ability of the system to detect BPA was linear above the IC\textsubscript{50} value, despite the addition of protein, lipid, and small molecule components.

Below this concentration, the signal was indistinguishable from background, likely due to surface passivation from proteins in the formula.

As a final experiment, we evaluated the ability of the system to detect EA from an everyday source without prior knowledge of the contaminants. In the literature, E-SCREEN assays have shown that certain “BPA-free” plastic baby bottles release EDCs upon microwave heating.\textsuperscript{24} We sought to replicate this experiment using the faster and lower volume electrochemical assay described herein. Prior to microwave heating the plastic bottle, the buffer had no observable EA. However, after microwaving for ten 2 min periods, the buffer in the plastic bottle had significant EA, comparable to 100 nM E2. In contrast, the buffer in a glass bottle contained no EA before or after microwaving (Figure 4c).

Through this work, we have developed a new approach for determining the estrogenic activity of endocrine disrupting compounds. By combining impedance spectroscopy based detection with the signal amplification provided by a lyophilized \textit{E. coli} scaffold, large responses in the charge transfer resistance of the electrode are observed, even in the presence of sub-ppb estradiol. The system provides the first reported sensor that responds broadly to all EDCs, and since it is based on inexpensive disposable electrode technology, it can be used in the field. The 10 \(\mu\text{L}\) sample size is far smaller than that needed for cell-based growth assays, and the readout is available in minutes, not days. Furthermore, the application of lyophilized \textit{E. coli} as a scaffold for our protein provides a new method of signal amplification, and is crucially important for reaching the low detection limits that these compounds require. The system also shows promising compatibility with complex sample matrices, such as infant formula. This new sensing approach should be applicable to other diverse families of compounds that bind to a single receptor, such as PPARγ, and current efforts in our laboratory are exploring these possibilities.

\section*{METHODS}

\textbf{Plasmid Preparation.} \textit{Monobody Encoding pSKB3 Vector}. The gene encoding for the ERα-estradiol selective monobody protein, a sequence adapted from Koide et al.,\textsuperscript{19,20} was synthesized by IDT Technologies with \textit{Bam}HI and \textit{Xho}I restriction sites at the 5’- and 3’-ends and subcloned into a pSKB3 vector—a variation of Novagen’s pET-28a vector with the thrombin site exchanged for a TEV proteolysis site.
insert and vector backbone were double digested (BamHI/ 
XhoI), heat inactivated at 80 °C for 5 min, ligated with 
QuickLigase (NEB) at a 5:1 molar ratio, and transformed into 
XL1Blue competent cells. Plating on kanamycin agar plates 
yielded individual colonies, which were cultured, DNA purified 
(NucleoSpin, MacheryNagel), and sequenced (Quintara 
BioSciences).

INPNC-ERα Encoding pSKB3 Vector. The synthetic gene 
(IDT Technologies) of ERα (organism, Homo sapiens; gene, 
ESR1, accession number P03372; residue number, 301–552) 
was subcloned with Nhel and NotI restriction sites at the 5’- 
and 3’-ends into a pSKB3 vector containing an N-terminal 
maltose binding protein (MBP). The resulting vector furnished 
the following amino acid sequence: MASS-(His)α-TEV-MBP- 
Linker-ERα (where “Linker” = Nα-EGASGSG).

The gene insert coding for the ice nucleation protein with 
the NC-terminal fusion (INPNC: fusion of the N-terminal 
membrane domain INPN and the C-terminal extracellular 
domain INPα) was synthesized by IDT Technologies with 
NcoI and NheI restriction sites at the 5’- and 3’-sites and was 
subcloned into the MBP-ERα pSKB3 vector above. The MBP 
gene was removed in the process. The resulting vector encodes 
for the following amino acid sequence: MAA-INPNα-RS-INPNα- 
SSNαLGSAGSG-ERα. The INPNC insert and vector backbone 
were double digested (NcoI/NheI), heat inactivated at 65 
°C for 15 min, ligated with QuickLigase (NEB) at a 5:1 molar 
ratio, and transformed into XL1Blue competent cells. Plating 
on kanamycin agar plates yielded individual colonies, which 
were cultured, DNA purified (NucleoSpin, MacheryNagel), and 
sequenced (Quintara BioSciences).

Protein Expression and Purification. Plasmids were 
transformed into E. coli BL21 (DE3) competent cells. Starter 
cultures (20 mL of LB, 50 mg/L kanamycin) were grown from 
single colonies overnight at 37 °C and used to inoculate 1 L of 
TB medium (50 mg/L kanamycin). Cultures were grown to an 
OD ∼ 0.5, cooled to 25 °C for 20 min, induced with 0.5 mM 
IPTG, and expressed overnight (18 h) at 25 °C. Cells were 
harvested by centrifugation for 15 min at 4000 rcf at 4 °C. The 
protein was purified directly without freezing.

Purification of Monobody Protein. The pellet was trans-
ferred to PBS buffer and centrifuged for 10 min at 4300 rcf. The 
resulting pellet was lysed in 30 mL of lysis buffer, referred to 
hereafter as buffer B (20 mM bicine, pH 8.5, 500 mM NaCl, 10 
mM imidazole), supplemented with one tablet of EDTA-free 
SigmaFast Protease Inhibitor (Sigma-Aldrich), 5 mM PMSF, 
and 2 mg of lysozyme. Without incubation, the resuspension 
was lysed with an Avestin C3 homogenizer followed by a 20 
min centrifugation at 24,000 rcf at 4 °C. The supernatant was 
filtered through a 40 μm Steriflip filter (Millipore) and loaded 
on to a 5 mL NiNTA column (Protino, Machery Nagel) 
connected to an Akta purifier preequilibrated with buffer B. 
The column was washed with 50 mL (10 column volumes) of 
20 mM bicine (pH 8.5), 500 mM NaCl, 10 mM imidazole, 10 
mM β-ME. The protein was eluted with 20 mM bicine (pH 
8.5), 500 mM NaCl, 250 mM imidazole, 10 mM β-Me. 
Imidazole was removed by exchanging against 20 mM bicine 
(pH 8.5), 500 mM NaCl, with a 10DG desalting column 
(BioRad). For purposes of lyophilization, the protein was 
directly exchanged against 20 mM bicine (pH 8.5) and 100 mM 
trehalose, followed by flash freezing with liquid N2 and 
lyophilization (Labconco) overnight. Typical protein yields 
are 800 μM (3 mL total) from a 1 L culture, with a purity of 
~98% by SDS–PAGE and LC–MS (ESI-TOF) (6224 TOF 
and 1200 series HPLC, Agilent Technologies).

Expression of Cell Surface Displayed INPNC-ERα. INPNC- 
ERα was expressed from single BL21 colonies. The proteins 
were grown in 50 mL of TB in the presence of 50 mg/L 
kanamycin. At an OD₆₀₀ ∼ 0.5 the culture was equilibrated to 
25 °C for 20 min, induced with 0.5 mM IPTG, and expressed 
overnight (18 h) at 25 °C. The cells were centrifuged for 5 min 
at 4000 rcf and resuspended either in M9 minimal medium for 
direct use or in 20 mM HEPES (pH 7.5) with 100 mM 
trehalose for lyophilization. E. coli cells were lyophilized by flash 
freezing 100 μL aliquots in liquid nitrogen at an OD₆₀₀ ∼ 0.1. 
The lyophilized samples were stored at either −20 °C or −80 
°C. Sodium azide (NaN₃) treated cells were incubated with 5 
mg/mL NaN₃ to induce toxicity. Chloramphenicol-treated cells 
were incubated with 10 μg/mL of chloramphenicol for 30 min, 
a concentration below toxicity but sufficient to inhibit protein 
synthesis.

Cell Viability of Lyophilized E. coli Cultures. The 
lyophilized sample produced from 100 μL at OD₆₀₀ = 0.1 was 
dissolved in 600 μL of M9 minimal medium, and 125 μL 
volumes were streaked onto kanamycin agar plates. No colonies 
were observed after 24 h; colonies observed after 48 h were 
counted and compared against equivalently plated cells 
stored from glycerol stocks.

Reconstitution of E. coli Cells. Aliquots of E. coli 
were reconstituted by dissolution in 100 μL of 20 mM HEPES (pH 
7.5) to an OD of 0.1. Cells were incubated on ice for 20 min 
prior to further dilution.

Determination of Surface-Expressed ERα. An estradiol– 
coumarin conjugate (prepared as described in ref 21) was 
added to a final concentration of 10 μM to either freshly 
harvested or lyophilized E. coli at an OD₆₀₀ of 0.01 in M9 
medium. Following a 20 min incubation, cells were purified 
from unbound E2–coumarin by spin filtration (10K, 5 min, 
5000 rcf). The fluorescence of the E2–coumarin labeled cells 
was measured and compared to a standard curve of estradiol– 
coumarin fluorescence in M9 medium. Using the concentration 
of estradiol–coumarin, the number of receptors per E. coli cell 
was estimated.

Electrode Preparation. Disposable gold electrodes (1.3 
mm diameter, cold annealed, DropSens) were preliminarily 
prepared in 0.5 M H₂SO₄ by scanning from 1.3 V to −0.2 V (vs 
internal reference, 100 mV scan rate, 9 scans). Electrodes were 
subsequently washed with Milli-Q water. Lyophilized mono-
body was diluted in 20 mM HEPES, 300 mM NaCl (pH 7.5) to 
a final concentration of 50 μM. A 10 μL portion was added 
the electrode surface. Electrodes were placed in humidifier 
boxes and incubated overnight at 4 °C. Prior to detection using 
the monobody-modified electrodes, the electrodes were 
rinsed with 3 aliquots of 100 μL of 20 mM HEPES (pH 7.5).

Incubation of Analytes. Following reconstitution of 
lyophilized E. coli samples, the cells were diluted to the desired 
final concentration (10⁶ cells/mL, although cells were tested at 
centrations ranging from 10⁵ to 10⁷) in 20 mM HEPES (pH 
7.5). For the detection of single endocrine disrupting chemicals 
(estriadiol, DES, GEN, BPA, or 4-NP), the chemical was 
dissolved in DMSO to a 1000X dilution of the final 
centration, such that the concentration of DMSO was 
constant at 0.1% v/v in solution with E. coli, including negative 
controls (in which either no cells or no EDC was added). 
The EDC of interest was incubated with the E. coli for 20 
in solution prior to application to the electrode. A 10 μL
portion of the E. coli solution containing the analyte compounds was then added to the electrode surface. E. coli solutions were incubated on the electrode surface for 20 min at ambient temperature in a humidifier box. The electrodes were subsequently rinsed with 3 aliquots of 100 μL of 20 mM HEPES (pH 7.5).

Electrochemical Impedance Spectroscopy. Electrochemical impedance spectroscopy (EIS) was performed with a Gamry Reference 600 potentiostat. The buffer consisted of 4 mM each of K3Fe(CN)6/K4Fe(CN)6 in 0.1 M KCl. Electrochemical measurements were acquired at the open circuit potential of the electrode and measured for 60 s prior to EIS. EIS measurements were made from 50,000 to 0.2 Hz with 10 points per decade and a 10 mV ac voltage. Electrochemical data analysis, including circuit modeling, was performed using the Gamry Echem Analyst software. Charge transfer resistance (R_CT) was derived from a constant phase element (CPE) with diffusion circuit model fit.

ASSOCIATED CONTENT

 Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.6b00322.

 Figures S1−S5, Table S1, and plasmid and insert sequences (PDF)

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Notes
The authors declare no competing financial interest.

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