

A High-Throughput Screening Assay of Endocrine-Disrupting Chemicals Using a Receptor-Modified Au-Electrode

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There is a great deal of concern that wildlife and human health have been adversely affected by anthropogenic chemicals that can disrupt normal endocrine homeostasis. In order to identify the binding ability of chemicals to human estrogen receptors (hER), we have constructed a biosensor which carries the hER ligand-binding domain on the surface. The receptor was expressed as an in-frame fusion with ten consecutive histidine residues using a bacterial system, and then the recombinant protein was immobilized on an Au-electrode via Ni(II)-mediated chemisorption using the histidine tag and thiol-modified nitrilotriacetic acid. This receptor-modified electrode was used to define the binding capacity of several xenoestrogens and anti-estrogen to the hER. The biosensor showed a linear response to the chemicals in a concentration-dependent manner. The order of the detection limits was: 17β-estradiol (10^{-10} M) > diethylstilbestrol (DES; 10^{-8} M), ICI 182780 (10^{-8} M), dibutyl phthalate (10^{-8} M), bisphenol A (10^{-8} M) > *p*-nonylphenol (10^{-6} M), testosterone (10^{-6} M). Compared to the traditional binding assay, our method has the advantage of being more feasible, due to the high-throughput screening assay for evaluating the binding ability of chemicals to the receptors.

Steroid hormones regulate the transcription of target genes by binding to specific intracellular receptors. For example, estrogen, a female sex steroid, exerts its physiological effect by binding to the hERs, which are members of the nuclear receptor superfamily of ligand-inducible transcription factors. The estrogen receptor regulates the differentiation, development, and maintenance of neural, skeletal, cardiovascular, and reproductive tissue.[1]

Recently, considerable attention has been focused on a certain number of environmental contaminants of industrial origin that may mimic the action of estrogen. These chemicals, called "endocrine-disrupting chemicals" (EDCs), may act to alter blood hormone levels or the subsequent action of hormones, including effects on hormone production, release, transport, and metabolism.[2] Consequently, it has been hypothesized that environmental exposure to these synthetic estrogenic chemicals and related endocrine-active compounds may be responsible for a global decrease in sperm counts, decreased male reproductive capacity, and breast cancer in women.[3] Although the detailed mechanisms of the disrupting actions of endocrine disruptors in biological systems remain elusive, certain chemicals bind to endogenous hormone receptors as well as natural steroids, subsequently disturbing normal endocrine function. Chemicals suspected of being endocrine disruptors are comprise of a wide variety of substances, both natural and man-made, including some substances that are widely used and are of great commercial value. In view of this alarming situation, there is a great need for an effective screening method for these chemicals.

In the last few years, several research groups have developed in vivo and in vitro screening assays for endocrine effects.[3–8] While it is clear that a spectrum of in vivo screens may be required to characterize a compound as an endocrine disruptor that can cause adverse effects in exposed organisms, in vitro assays are required to define the molecular mechanisms responsible for these effects.[9] In particular, in view of the enormous number of compounds that must be examined in order to get an overview of the potential estrogenic chemicals present in our environment, a rapid, sensitive, and reliable in vitro screening method is required.

The combination of high specificity in biological recognition with electrochemical detection is potentially a very useful technique for creating sensors with applications in food, medicine, and the environment. We have previously demonstrated a novel bioaffinity sensor aimed at the detection of steroid hormones.[10,11] This biosensor system was based on the specific binding of estrogen to its receptor immobilized on a gold disk electrode (Fig. 1). Unfortunately, affinity reactions between the nuclear receptors and their ligands are not directly linked to an electrochemical response. Thus, we used the concept of an 'ion-channel mechanism'[12–15] with a redox marker in the biosensor. In the present study, we applied this bioaffinity sensor to examine the estrogenic potency of six chemicals including endogenous steroid hormones, synthetic hER agonists, estrogenic antagonists, and anthropogenic compounds.

Experimental

Reagents. 17β-Estradiol (>97%) and testosterone (>97%) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). DES (>99%) and dibutyl phthalate were purchased from Sigma (St. Louis, USA). Bisphenol A (>99%) and *p*-nonylphenol (technical grade) were supplied from Aldrich (Milwaukee, USA). ICI 182780 was purchased from Tocris Cookson Ltd. (Ballwin, USA). All other chemicals were reagent grade, obtained from commercial sources, and used without further purification.

Expression and Purification of Human ER Ligand-Binding Domain. Plasmid pEThER-LBD, the bacterial expression vector containing the human ER ligand-binding domain (hER-LBD, amino acids 302–553) gene, fused with the N-terminal polyhistidine

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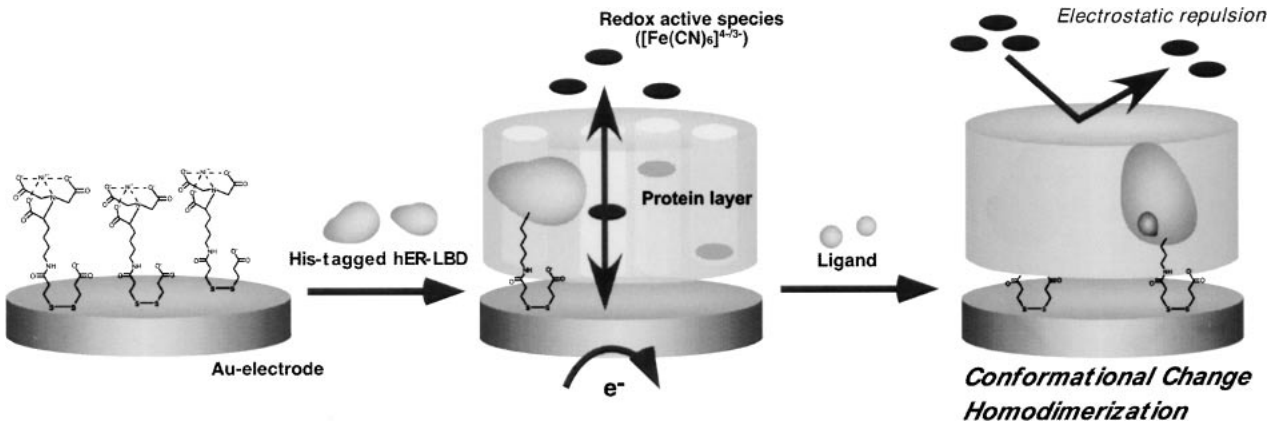

Fig. 1. Schematic illustration of the electrochemical sensing system for endocrine-disrupting chemicals based on the ligand–receptor interaction.

sequence, has been constructed previously.[10] This vector was expressed in hER-LBD in *Escherichia coli* BL21(DE3)pLysS cells (Novagen) using the T7 expression system. *E. coli* BL21(DE3)-pLysS containig pEThER-LBD were grown overnight at 37 °C in 4 mL of Luria Broth supplemented with 200 μg/mL ampicillin and 34 μg/mL chloramphenicol. After this, the cells were diluted 10-fold in fresh medium containing 50 μg/mL ampicillin and 30 μg/mL chloramphenicol, and the outgrowth was allowed to proceed to an optical density of 1.0 at 600 nm. Recombinant protein production was initiated by the addition of isopropyl thiogalactopyranoside (IPTG; Wako Pure Chemical Industries Ltd.) to a final concentration of 1 mM, followed by incubation for another 4 h. The cells were pelleted by centrifugation at 4 °C. The pellet was resuspended in 8 mL of lysis buffer (50 mM NaH_2PO_4–NaOH (pH 8.0), 5 mM 2-mercaptoethanol, 0.1 mM PMSF, 5 mM NaMBS). The resulting suspension was frozen at −80 °C, and then thawed at 37 °C. Following sonication on ice (45 s, 200 W), DNase I and RNase A were added to give final concentrations of 5 μg/mL and 1 μg/mL, respectively, and the mixture was incubated for 30 min at 4 °C. NP-40 was added to the final concentration of 0.1% and NaCl to a final concentration of 300 mM. Cell fragments and insoluble material were removed by centrifuging (20 min, 20000*g*) at 4 °C. Purification of the recombinant protein was performed by affinity chromatography under native conditions. The supernatant (soluble material) was loaded on a 5 mL Hitrap™ chelating column (Amersham Pharmacia Biotech). The column was washed with 20 mL of wash buffer (50 mM NaH_2PO_4–NaOH (pH 6.0), 300 mM NaCl, 5 mM 2-mercaptoethanol, 0.1 mM PMSF, 5 mM NaMBS, 10% glycerol) followed by 20 mL of wash buffer containing 80 mM imidazole. The His-tagged hER-LBD was eluted by wash buffer containing 500 mM imidazole. Fractions containing the hER-LBD were collected and loaded on a PD-10 desalting column. The hER-LBD was eluted with store buffer (50 mM Tris–HCl (pH 7.4), 500 mM NaCl, 1 mM DTT, 0.1 mM PMSF). The protein concentration was determined using the Bradford protein assay (Dojindo, Japan) with bovine serum albumin as a standard.

Cell lysate fractions, purified with hER-LBD, were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using 12% gel according to standard protocol. The gels were either stained with Coomassie Brilliant Blue or transferred to polyvinylidene difluoride membrane (Millipore) for Western blotting. After transfer for Western blotting, the filter was blocked and incubated with the anti-His HRP conjugate (Qiagen). The bound antibody was detected with Super Signal West Pico® chemiluminescent reagents (Pierce) using a Night OWL® molecular light imager (Berthold).

Immobilization of hER-LBD onto the Au Disk Electrode. The disulfide-NTA, 3,3′-dithiobis[*N*-(5-amino-5-carboxypentyl)-propionamide-*N*′,*N*′-diacetic acid] (DOJINDO), was immobilized on an Au electrode surface via chemisorption.[16] A polished Au disk electrode (1.6 mm diameter, Bioanalytical Systems) was immersed in 10 mM disulfide-NTA in chloroform for 3 h at 4 °C. The electrode was then washed by chloroform and was dried with nitrogen. The NTA-modified electrode was immersed in 1 M NaOH overnight at 4 °C for the hydrolysis treatment of activated-ester moieties. The electrode was washed in distilled water and then immersed in 0.1 M $NiSO_4$ for 10 min at room temperature. The Ni–NTA-modified electrode was immersed in 4 μM hER-LBD solution for 10 min at 4 °C and then rinsed with 10 mM Tris–HCl (pH 7.4) aqueous solution containing 100 mM KCl. Finally, the modified electrode was treated with 10 mM 6-mercaptohexanol aqueous solution at 4 °C for 20 min to avoid a non-specific adsorption of ligand molecules. In order to confirm the validity of this protein modification method, quartz crystal microbalance (QCA917, Seiko EG&G) measurements were carried out before and after the modification procedure with an Au-coated quartz crystal (At-cut 9 MHz, 5 mm diameter, roughness factor 1.71) instead of an Au disk electrode.

Electrochemical Measurements with Receptor-Modified Au Electrode. We examined the binding behavior of the hER-LBD to its ligand on the electrode by CV measurements. The CV measurements were performed in 10 mM Tris–HCl (pH 7.4) containing 100 mM KCl and 5 mM $K_{3/4}[Fe(CN)_6]$ at room temperature with a conventional three-electrode system consisting of a Pt wire counter, a standard Ag/AgCl (saturated KCl) reference electrode and the receptor-modified Au electrode (3 mm diameter; Bioanalytical Systems) using a Bioanalytical System Model CV-50W potentiostat.

Results and Discussion

The ER is comprised of several functionally-distinct domains.[17,18] The N-terminal A/B domain contains the transcription activation function. The highly conserved C domain is the site of DNA binding to estrogen-response elements, whereas the D domain appears to function as a hinge region. Domains E and F, at the C terminus of the receptor, are the regions of ligand binding and recognition and contain the ligand-dependent

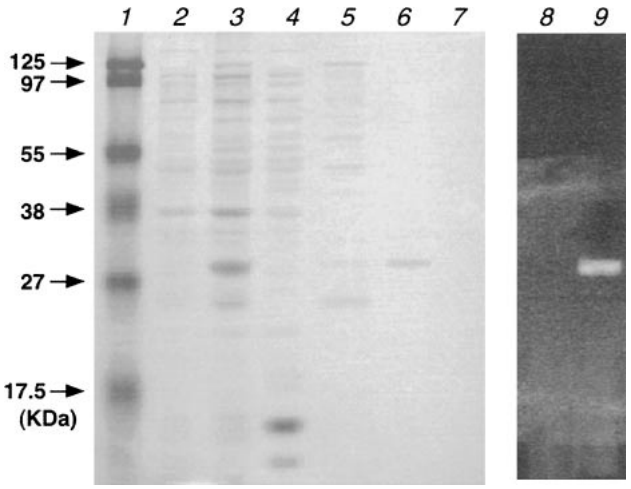

Fig. 2. Analysis of the expression and affinity purification of the hER-LBD synthesized in *E. coli*. A, Coomassie Blue-stained 12% SDS-polyacrylamide gel of bacterial extracts. B, Western immunoblot analysis of duplicate samples as shown in A. Lane 1, protein MW marker; lanes 2 and 8, uninduced crude extract; lanes 3 and 9, IPTG-induced crude extract; lane 4, flow-through fraction; lane 5, 80 mM imidazole wash, lane 6, 250 mM imidazole elution; lane 7, 50 mM EDTA elution.

transactivation function-2.[19] Initial attempts to express the full length hER in bacteria suggested that the expression was relatively low and almost all of the protein was produced in an insoluble form. Consequently, we constructed an expression vector, pEThER-LBD, in which the ligand binding domain of hER could be expressed in *E. coli* as the N terminus of an in-frame fusion with ten consecutive histidine residues.

E. coli [BL21(DE3)pLysS] were transformed with pEThER-LBD and then grown in the presence or absence of 1 mM IPTG. As expected, the SDS-PAGE analysis of the total cell extracts showed the recombinant fusion protein hER-LBD with the predicted molecular weight (32 kDa) which appeared only in cell extracts prepared from IPTG-treated cells (Fig. 2A, lane 3). The fusion proteins were never observed in extracts from IPTG untreated cells (Fig. 2A, lane 2). We confirmed that this induced protein was indeed histidine fusion protein by Western immunoblotting analysis using a monoclonal antibody specific for five consecutive histidine residues. As shown in Fig. 2B, a protein with the required epitope and the expected size for the expressed hER-LBD was present only in the IPTG treated cell.

The fusion protein was purified by affinity chromatography under native conditions. The soluble fraction of the crude lysate was loaded on a NTA-carrying resin. hER-LBD containing a histidine affinity tag was effectively bound to the resin (Fig. 2A, lane 3). The removal of background proteins and the elution of histidine-tagged protein from the column was achieved by the addition of imidazole, which competes with the tagged proteins for binding sites on the NTA resin, as described under the experimental section (Fig. 2A, lanes 4, 5). In this manner, the hER-LBD was obtained as a nearly homogenous species and did not contain any breakdown products. The concentration of the purified fusion protein was determined by the method of Bradford, and ranged between 1.3 and 2.0 mg/L-cell culture.

The amount of immobilized recombinant protein on the gold surface was monitored by QCM measurement. The QCM technique is a suitable method for detecting the adsorption of biomolecules at the quartz surface owing to its sensitivity to changes in mass and viscoelastic properties.[19,20] In this study, the recombinant protein was immobilized on Au-electrodes with Ni(II)-mediated chemisorption using the histidine tag and thiol-modified NTA.[11] The NTA-modified quartz plate, which was prepared by a similar procedure as that of the NTA-modified Au disk electrode, was soaked in an aqueous buffer solution of hER-LBD. After washing the quartz plate with distilled water, the frequency change was measured at room temperature. The resulting frequency decrease was 89.5 MHz compared with the bare quartz plate. According to Sauerbrey's equation,[21] the immobilized amount of hER-LBD was calculated to be 8.9 pmol cm^{-2} from the frequency decrease after protein binding. In fact, it was estimated that hER-LBD was present every ca. 50 Å on the gold surface by the present method.

The interaction between the recombinant receptor and its ligand molecules on the Au disk electrode surface was evaluated by electrochemical measurements. The peak currents due to the reversible redox reaction of the marker ions decreased on the hER-LBD modified gold disk electrode compared with the bare one. As shown in our previous research, this peak current depression is explained by a decrease in the local concentration of the marker ions near the electrode, mainly due to the steric barrier arising from the electrode modifications.[13–15] Figure 3 shows the cyclic voltammograms of the electrode in the absence and presence of 17β-estradiol, a typical female sex steroid. The redox current was significantly suppressed by 17β-estradiol in a concentration-dependent manner in the hER-LBD modified electrode system. The electrochemical responses

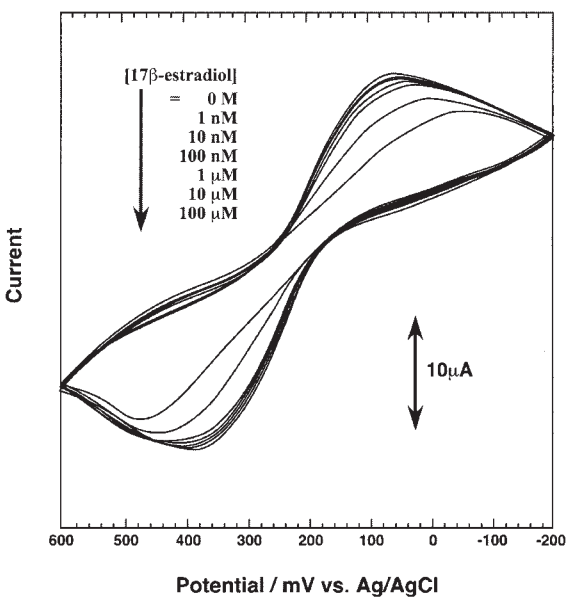

Fig. 3. Concentration dependence of estrogen to redox currents of hexacyanoferrate(II)/hexacyanoferrate(III) redox couple on the protein modified Au electrode. All measurements were performed in 10 mM Tris buffer, pH = 7.4, [KCl] = 100 mM, $[Fe(CN)_6]^{4-/3-}$ = 5 mM, at 25 °C.

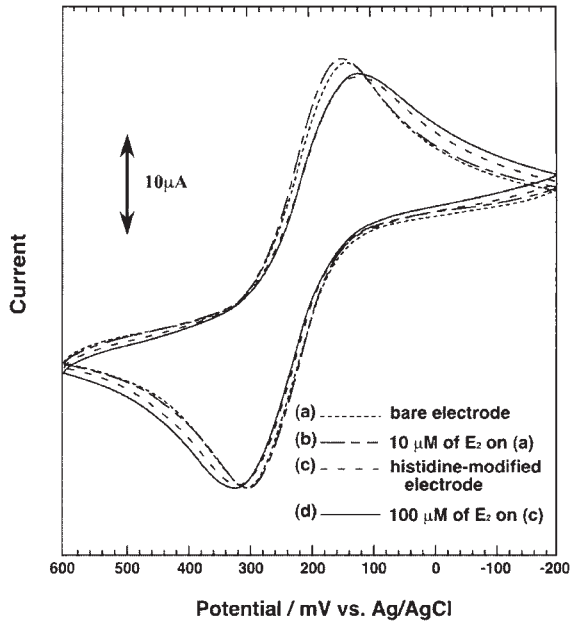

Fig. 4. Cyclic voltammograms of a protein-unmodified Au electrode. (a), gold disk electrode; (b), after a treatment of 10 μM of 17β-estradiol on the bare electrode; (c), histidine-modified electrode; (d), after a treatment of 100 μM of 17β-estradiol on the histidine-modified electrode. All measurements were performed in 10 mM Tris buffer, pH = 7.4, [KCl] = 100 mM, $[Fe(CN)_6]^{4-/3-}$ = 5 mM, at 25 °C.

achieved an equilibrium situation within 5 minutes. On the other hand, unmodified-receptor electrodes (bare and histidine-modified electrodes instead of the hER-LBD) showed no response to the 17β-estradiol, even if 100 μM of 17β-estradiol was added to the testing solution (Fig. 4). This phenomenon, therefore, was attributed to the immobilized hER-LBD that complexed with the ligand molecule on the electrode surface and perturbed the electrical contact and electron transfer rate between the redox probe and the electrode surface. Although it is not clear how the complexation influences the amperometric transduction of the redox marker at the electrode surface, ligand-inducible conformational changes in the hER-LBD on the electrode surface may affect this peak current depression. It is known that the transcriptional response to hormones is rooted in conformational changes of hormone receptors induced by specifically-bound ligands. In the case of hER, complexation with ligands directly induces conformational changes, and the receptor is then capable of either binding to DNA as a homodimer or interacting with other transcriptional factors. In particular, the ligand-binding domain of the hER, located in a C-terminal region of approximately 250 amino acids, plays an important role in these functions, identified by functional analyses and structural studies of the receptors.[18,22–24] Interestingly, Witkowska et al. observed that positive charges on the surface of the hER ligand-binding domain decreased dramatically with ligand-binding in the presence of 17β-estradiol under physiological conditions.[25] It seems reasonable to suppose that the electrostatic properties of the protein layer on the electrode surface were similarly altered by complexation with estrogen. In fact, suppression of the electrochemical reaction of the negatively-charged marker ion, the hexacyanoferrate(II)/hexacyanoferrate(III) redox couple, supports our view (Fig. 1).[10] These data suggest that the biosensor presented here is applicable to the evaluation of binding activities of chemicals toward the human estrogen receptor.

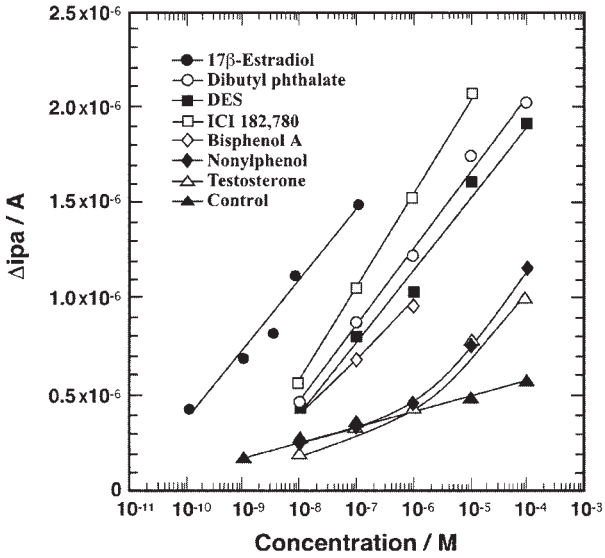

Fig. 5. Concentration-dependent changes in the anodic peak current (Δipa) on the Au electrode modified with the hER-LBD. ●, 17β-estradiol; ○, dibutylphthalate; ■, diethylstilbestrol; □, ICI 182780; ◇, bisphenol A; ◆, nonylphenol; △, testosterone; ▲, DMSO as a control. All measurements were performed in 10 mM Tris buffer, pH = 7.4, [KCl] = 100 mM, $[Fe(CN)_6]^{4-/3-}$ = 5 mM, at 25 °C.

The binding activities of other chemicals to the hER-LBD were determined using this biosensor. The chemicals studied were: bisphenol A, dibutyl phthalate, *p*-nonylphenol, DES, ICI 182780, and testosterone. Dimethyl sulfoxide (DMSO) was used as the solvent control. The peak currents due to the redox reaction of the marker ions in the presence of these compounds decreased in a concentration-dependent manner similar to the case of 17β-estradiol described above. In contrast, the unmodified electrodes showed no response to the chemicals (data not shown). Figure 5 shows the relationship between the absolute values of the diminution of the anodic peak current (Δipa) and the concentration of these compounds. The hER-LBD-modified electrode showed a good response to the compounds over a wide range of concentrations. 17β-Estradiol was the most sensitive ligand for the sensor. Our sensor showed a linear response to 17β-estradiol in the range of 10^{-10} to 10^{-7} M. The sensitivity of this electrochemical sensing system based on a ligand-receptor interaction was comparable to the case of other in vitro screening methods, such as competitive binding assays using radioactive estradiol[26] or a yeast two-hybrid system.[5,27] Bisphenol A and dibutyl phthalate, which have been shown to bind with hER and disturb normal endocrine function, were as effective as ICI 182780, a estrogenic antagonist, and DES, a synthetic hER agonist. *p*-Nonylphenol and testosterone were active at a high concentration. On the other hand, DMSO as a control had no significant effect on the sensor under experimental conditions. It is known that DES binds with hER as strongly as 17β-estradiol,[5,9] however, the hER-LBD-modified electrode showed a lower sensitivity to DES compared with

17β-estradiol. The results may be due to an incomplete conformational change of the receptor anchored on the electrode surface, because it is known that DES induces different conformational changes involving the repositioning of helix 12, the closest C-terminal helix of the hER-ligand binding domain, from other agonists, including 17β-estradiol.[23]

In conclusion, the novel biosensor based on the ligand-receptor interaction reported here is applicable as a convenient tool for the evaluation of the hER binding ability of chemicals. This electrochemical binding assay offers several advantages over other binding technologies. First, our sensor system requires no chemical modification or radio-labeling of the test compounds for detection. Secondly, this method can determine the direct interaction between hER and estrogenic substances in contrast with competitive binding assays using a labeled tracer. Thirdly, the sensor can detect electrochemical signals after as little as 5 minutes of incubation. Furthermore, separation procedures including filtering, precipitation, and/or centrifugation to measure the tracer's bound/free ratio are eliminated, thereby reducing the hands-on laboratory time and artifactual loss of signal. In order to define the estrogenic potency of a number of anthropogenic chemicals, there is a great need for a rapid screening assay for their detection. While it is necessary to investigate into the re-use of the receptor modified electrode and the influence of coexisting materials in the measurement solution, the electrochemical binding assay has the potential to be a reliable and high-throughput screening method for new ligands for the hER. This approach for predicting hER binding may be applied to other receptors, such as the androgen receptor, thyroid hormone receptor, or dioxin receptor, and so on. We will report on the electrode modified with these receptors in the near future. These sensors will be used not only as a large-scale screening tool for EDCs but also as an effective method for screening purposes in drug discovery or clinical diagnosis.

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