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An electrochemical device for the assay of the interaction between a dioxin receptor and its various ligands

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Abstract—Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates the toxic and biological effects of a variety of chemicals. Although a significant amount of information is available with respect to the planar aromatic hydrocarbon AhR ligands, information on the actual spectrum of chemical structures that can bind to and activate the AhR is insufficient. In order to determine the binding affinities of chemicals to the human AhR (hAhR), we constructed an electrochemical system which carries the hAhR ligand-binding domain on the electrode surface. The recombinant hAhR ligand-binding domain that was expressed in *Escherichia coli* using a T7 expression system was immobilized on a gold electrode. The specificity of this biosensor based on a ligand–receptor interaction was comparable to other in vitro screening methods. The receptor-modified electrode can rapidly detect the binding of ligands to hAhR. The electrochemical measurement can be carried out within just 5 min. This electrochemical screening system is rapid, low in cost, and adaptable to high-throughput applications without sacrificing either sensitivity or selectivity.

1. Introduction

AhR is a ligand-activated transcription factor that regulates many genes involved in xenobiotic metabolism, cellular proliferation and differentiation.[1,2] AhR transforms to a DNA-binding form by a series of processes initiated by ligand binding. Subsequent steps include dissociation of several proteins that associate with the inactive receptor, nuclear translocation, and dimerization with the Ah receptor nuclear translocator (ARNT)[3,4] The AhR is an orphan receptor whose physiological ligands are unknown, however, it is known that some halogenated aromatic compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and polyaromatic hydrocarbons (PAHs) such as β-naphthoflavone (β-NF) and benzo[*a*]pyrene (B[*a*]P) can bind to this receptor. PAHs are a class of toxic organic chemicals comprising hundreds of congeners that are ubiquitous in the environment and in foodstuffs. They are produced and released into the environment by the incomplete combustion of fossil fuels, oil spills, and also by various industrial processes. A number of PAHs have shown carcinogenicity in experimental animals following oral, respiratory, dermal or subcutaneous administration. Most carcinogenic PAHs are metabolically activated, and they activate the AhR, which might play a role in promoting carcinogenicity.[5–7]

Although numerous xenobiotic and biological compounds are known to interact with AhR, the full spectrum of structural chemical classes that can interact with the AhR remains to be elucidated. Large-scale investigation of the specificity of binding ligands for AhR needs a rapid, sensitive, and reliable screening method. While chemical analyses such as GC–MS are sensitive and specific, they are expensive and provide little information on the actual or potential biological activities of the contaminants. Several research groups have developed in vitro screening assays for PAHs and dioxin-like compounds based on biological systems.[8–11] The AhR-chemically activated luciferase gene expression (CALUX) assay is based on eukaryotic cells stably transfected with a luciferase reporter gene, which is an effective and inexpensive tool.[12] Unfortunately, these cell culture bioassays are time consuming and have relatively low reliabilities, so they are unsuitable for use as high-throughput screening methods for new AhR ligands. In a previous report, we demonstrated a novel

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bioaffinity sensor based on the specific binding of estrogen to its receptor immobilized on a gold disk electrode.[13,14] This electrochemical sensor system requires no chemical modification or radiolabeling of the test chemicals for detection, and thus it does not need separation procedures to measure the labeled tracer's bound/free ratio. As a result, it can reduce the hands-on laboratory time required, as well as artifactual loss of signal. Furthermore, the electrochemical signals for the detection can be obtained after only 5 min of incubation using a biosensor. In this paper, we extend this methodology to the screening of AhR-binding ligands. Thus, we expressed recombinant human AhR in bacteria using a histidine-tag fusion system, and immobilized the receptor on a Au electrode. This bioaffinity sensor was then applied to the detection and relative quantitation of AhR receptor ligands.

2. Results and discussion

2.1. DNA construction and expression of recombinant hAhR

Plasmid pGEXhAhR$_{102–446}$, the bacterial expression vector containing the his-tag gene fused in frame with the ligand binding region of hAhR (amino acids 102–446), was cloned by PCR. The human cDNA library from Hela endocervical carcinoma cells was used as a PCR template. The primers for AhR$_{102–446}$ were: 5′-CGGCCGAAGCACCACCACCACCACCACAGAG-CACAAATTTCAGAGAA-3′ and 5′-GCGGCCGCTA-GAGTGGATGTGGTAGC-3′, and they contained *Eag*I and *Not*I, respectively (restriction sites underlined). The PCR-generated 1.07-kb fragment was digested with *Eag*I, and the resulting DNA fragment was subcloned into *Eag*I-digested pGEX-5X vector (Amersham Pharmacia Biotech). Correct insertion of the fragment was verified by DNA sequencing. Plasmids were amplified and purified by standard techniques.

The resulting vector was allowed to express the hAhR$_{102–446}$ in *Escherichia coli* BL21(DE3)pLysS cells (Novagen) using a T7 expression system. *E. coli* BL21(DE3)pLysS containing pGEXhAhR$_{102–446}$ were grown overnight at 37 °C in 4 mL of Luria Broth supplemented with 200 μg/mL ampicillin and 34 μg/mL chloramphenicol. After overnight growth, the cells were diluted 100-fold with fresh medium containing 200 μg/mL ampicillin and 34 μg/mL chloramphenicol, and the outgrowth was allowed to proceed to an optical density of 0.6 at 600 nm. Production of the recombinant protein was initiated by the addition of isopropyl thiogalactopyranoside (IPTG; Wako Pure Chemical Industries Ltd) to a final concentration of 1 mM, and incubation was continued for another 5 h at 27 °C.

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 β-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 and 1 μg/mL, respectively. The mixture was incubated for 30 min at 4 °C. Then NP-40 and NaCl were added to the solution to give final concentrations of 0.1% and 300 mM, respectively. Insoluble material was removed by centrifuging (20 min, 20,000*g*) at 4 °C. Purification of the recombinant protein was performed by affinity chromatography under non-denaturing 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 washing buffer (50 mM NaH_2PO_4–NaOH (pH 6.0), 300 mM NaCl, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 5 mM NaMBS, 10% glycerol) followed by 20 mL of washing buffer containing 80 mM imidazole. The His-tagged hER-LBD was eluted with washing buffer containing 500 mM imidazole. Fractions containing the hAhR$_{102–446}$ were collected and loaded on a PD-10 desalting column. The hAhR$_{102–446}$ was eluted by the store buffer [50 mM Tris–HCl (pH 7.4), 500 mM NaCl, 1 mM DTT, 0.1 mM PMSF]. Cell lysate fractions, purified with hAhR$_{102–446}$, were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using 12% gel according to standard protocol. The gels were stained directly with Coomassie Brilliant Blue or transferred to a polyvinylidene difluoride membrane (Millipore) for Western blotting.

2.2. Electrode modification and cyclic voltammetry (CV)

Histidine-tagged hAhR$_{102–446}$ was immobilized onto an Au electrode via metal (Ni^{2+}) complexation. 3,3′-Dithiobis[*N*-(5-amino-5-carboxypentyl)propionamide-*N*′,*N*′-diacetic acid] dihydrochloride (disulfide-NTA) was purchased from Dojindo lab. (Japan). The disulfide-NTA was immobilized onto an Au electrode surface via chemisorption. In order to achieve this a polished Au disk electrode (1.6 mm diameter, Bioanalytical Systems) was immersed in 30 μM disulfide-NTA in aqueous solution for 10 min at room temperature. The electrode was then washed with 50 mM HEPES-KOH (pH 7.2) containing 100 mM KCl. The NTA-modified electrode was immersed in 0.1 M $NiSO_4$ for 10 min at room temperature, and then immersed in 4 μM hAhR$_{102–446}$ solution for 10 min at 4 °C followed by washing with 50 mM HEPES-KOH (pH 7.2) containing 100 mM KCl. A human estrogen receptor ligand-binding domain (hER-LBD) was synthesized and immobilized on gold disk electrode as mentioned in our previous report.[15] This hER-LBD modified electrode was used as control electrode.

Cyclic voltammetry and differential pulse voltammetry were performed using a Bioanalytical Systems Co. Model ALS410 potentiostat. A platinum wire and a standard Ag/AgCl (saturated KCl) electrode were used as a counter and a reference electrode, respectively. β-NF and B[a]P (>97%) were purchased from Aldrich (Milwaukee, USA). In order to confirm the validity of this protein modification method, quartz crystal microbalance (QCM, USI Q-200 system) measurements were carried out before and after the modification procedure with an Au-coated quartz crystal (At-cut 9 MHz, 5 mm diameter) instead of an Au disk electrode.

The hAhR is comprised of several functionally-distinct domains.[16,17] The N-terminal basic helix–loop–helix (bHLH) region interacts with the AhR nuclear translocator (ARNT) and hsp90 and is involved in DNA binding. This region also contains amino acid sequences which are important for both nuclear localization and export of AhR. The AhR PAS domain contains two structural repeats which are involved in AhR/ARNT dimerization (PAS A), and ligand and hsp90 binding (PAS B). The C-terminal Q-rich domain contains the ligand-dependent transcriptional activity of hAhR. In this study, we constructed an expression vector, pGEXhAhR$_{102–446}$, in which the PAS domain of hAhR could be expressed in *E. coli* as the N-terminus of an in-frame fusion with six consecutive histidine residues.

Plasmid pGEXhAhR$_{102–446}$ was transformed into *E. coli* strain BL21(DE3)pLysS and gene expression was induced using IPTG at 27 °C. The expressed protein was purified by affinity chromatography on Ni(II)-NTA carrying resin and then analyzed by SDS-PAGE. A typical protein-expression and purification result is shown in Figure 1. The hAhR$_{102–446}$ with the predicted molecular weight (68 kDa) appeared only in cell extracts prepared from IPTG-treated cells (Fig. 1, lane 3). The fusion protein was never observed in extracts from IPTG-untreated cells (Fig. 1, lane 2). The affinity chromatography was carried out under non-denaturing conditions. The soluble fraction of a crude lysate was loaded on an NTA-carrying resin. hAhR$_{102–446}$ containing a histidine affinity tag was bound effectively to the resin. The removal of background proteins and the elution of histidine-tagged protein from the column was achieved by the addition of 80 and 500 mM imidazole, which competes with the tagged proteins for binding sites on the NTA resin (Fig. 1, lanes 4–6). In this manner, the hAhR$_{102–446}$ was obtained as a nearly homogenous species, and did not contain any breakdown products. The concentration of the purified recombinant hAhR was 1.0 mg/L-cell culture.

The histidine tag, located at the N-terminus of the hAhR$_{102–446}$, is uncharged at physiological pH and generally does not affect the folding of the protein. The histidine-tagged hAhR$_{102–446}$ can form a chelated complex with Ni(II)-NTA. Thus, this complex was immobilized on the Au-electrode surface via Ni(II)-NTA, which bound to Au with the disulfhydryl moiety via chemisorption.[13,14] The immobilization of the recombinant protein was examined by using cyclic voltammetry at 25 m Vs^{-1} in sample buffer [50 mM HEPES-KOH (pH 7.2), 100 mM KCl] containing 5 mM of both $K_4Fe(CN)_6$ and $K_3Fe(CN)_6$ at 4 °C. The peak currents due to the reversible redox reaction of the marker ions decreased on the protein-modified electrode compared with the bare one (Fig. 2). This phenomenon was attributed to immobilized hAhR$_{102–446}$ that suppressed the electrical contact and electron transfer rate between the redox probe and the electrode surface.

The amount of immobilized hAhR$_{102–446}$ on the gold surface was estimated by QCM measurement. The QCM technique is a sensitive surface sensor that is capable of measuring nanogram levels of mass change on the quartz surface. The NTA-modified quartz plate, which was prepared by a similar procedure to that for the NTA-modified Au disk electrode, was soaked in the aqueous buffer solution of hAhR$_{102–446}$. After washing the quartz plate with distilled water, the frequency

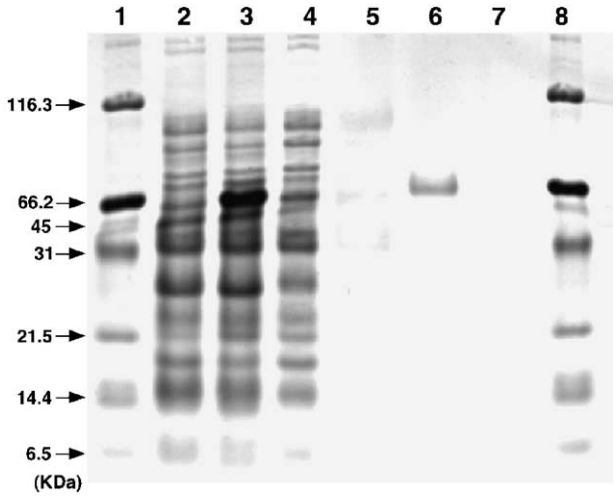

Figure 1. Analysis of the expression and affinity purification of the hAhR$_{102–446}$ synthesized in *E. coli*. Coomassie Blue-stained 12% SDS-polyacrylamide gel of bacterial extracts. Lanes 1 and 8, protein MW marker; lane 2, uninduced crude extract; lanes 3, IPTG-induced crude extract; lane 4, flow-through fraction; lane 5, 80 mM imidazole wash, lane 6, 250 mM imidazole elution; lane 7, 50 mM EDTA elution.

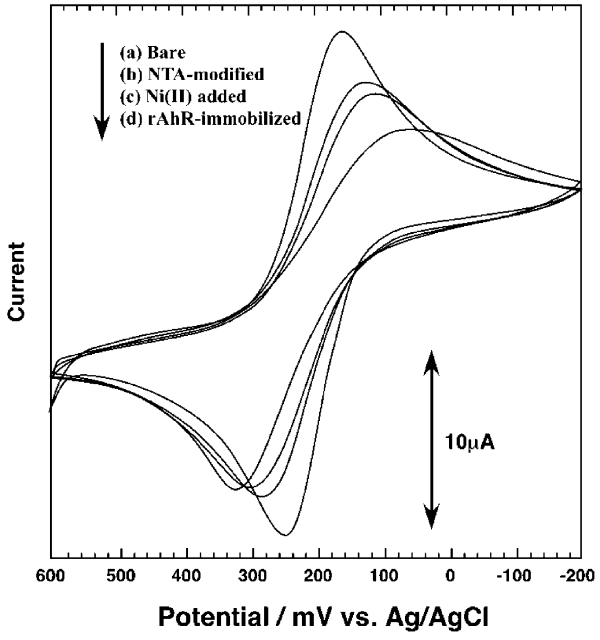

Figure 2. Cyclic voltammograms of the protein-modified Au electrode: (a), bare gold disk electrode; (b), after treatment of a disulfide-NTA; (c), after treatment of Ni(II); (d), after treatment of the electrode with the histidine-tagged hAhR$_{102–446}$. All measurements were performed in 10 mM Tris buffer, pH = 7.4, [KCl] = 100 mM, $[Fe(CN)_6]^{4-/3-}$ = 5 mM, at 25 °C .

change was measured at room temperature. According to Sauerbrey's equation,[18] the immobilized amount of $hAhR_{102–446}$ was calculated to be 4.7 pmol cm^{-2}, from the frequency decrease after protein binding. This means that, by the present method, $hAhR_{102–446}$ was immobilized every ca. 60 Å on the gold surface.

The $hAhR_{102–446}$-modified Au electrode was applied to the electrochemical detection of PAHs on the electrode surface. Figure 3 shows the cyclic voltammograms of the electrode in the absence and presence of β-NF. The redox current was suppressed by the β-NF in a concentration-dependent manner. As shown in Figures 4 and 5, the unmodified and hER-LBD modified electrode did not respond to the β-NF. Thus, the results demonstrate that the current depression was caused by complex formation between $hAhR_{102–446}$ and β-NF on the electrode surface. Figure 6 shows the relationship between the diminution of the anodic peak current (Δipa) and the concentration of β-NF. The $hAhR_{102–446}$-modified elec-

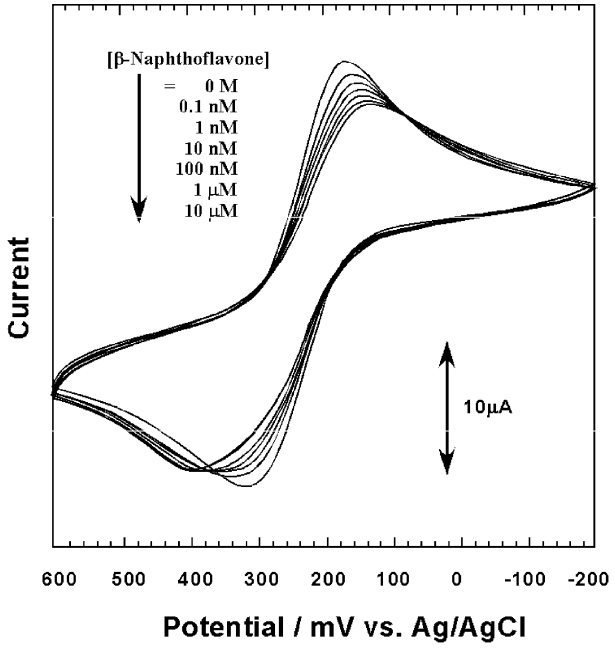

Figure 3. Concentration dependence of β-NF on redox currents of ferrocyanide/ferricyanide redox couple on the receptor-modified Au electrode. All measurements were performed in 10 mM Tris buffer, pH = 7.4, [KCl] = 100 mM, $[Fe(CN)_6]^{4-/3-}$ = 5 mM, at 25 °C.

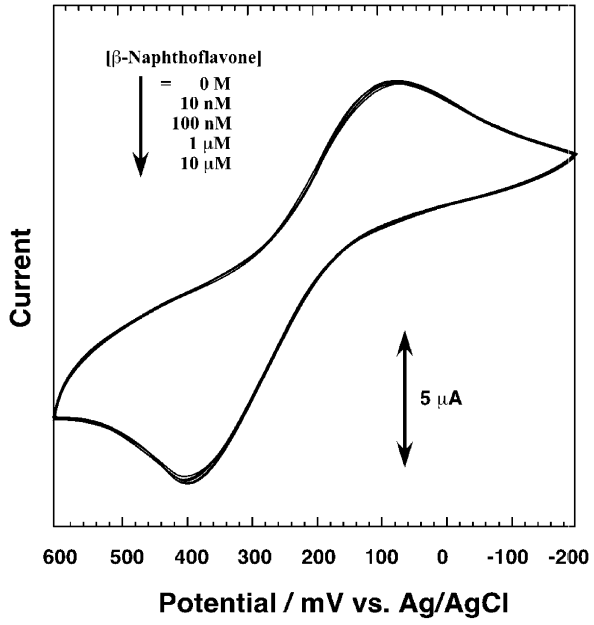

Figure 5. Cyclic voltammograms of hER-LBD modified Au electrode in the absence and presence of β-NF. All measurements were performed in 10 mM Tris buffer, pH = 7.4, [KCl] = 100 mM, $[Fe(CN)_6]^{4-/3-}$ = 5 mM, at 25 °C.

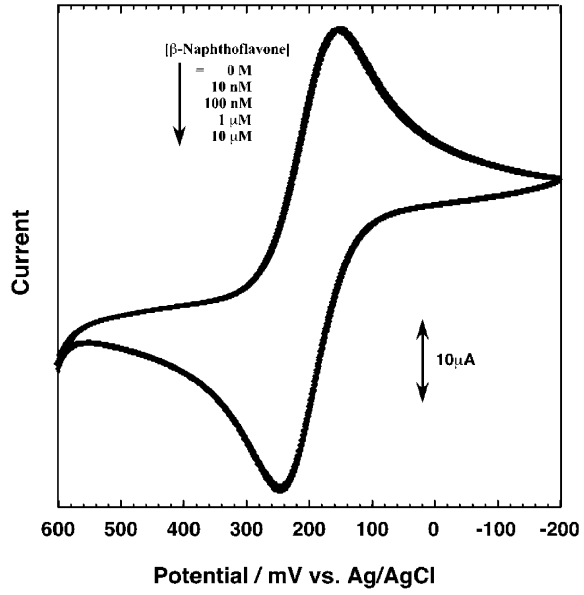

Figure 4. Cyclic voltammograms of unmodified Au electrode in the absence and presence of β-NF. All measurements were performed in 10 mM Tris buffer, pH = 7.4, [KCl] = 100 mM, $[Fe(CN)_6]^{4-/3-}$ = 5 mM, at 25 °C.

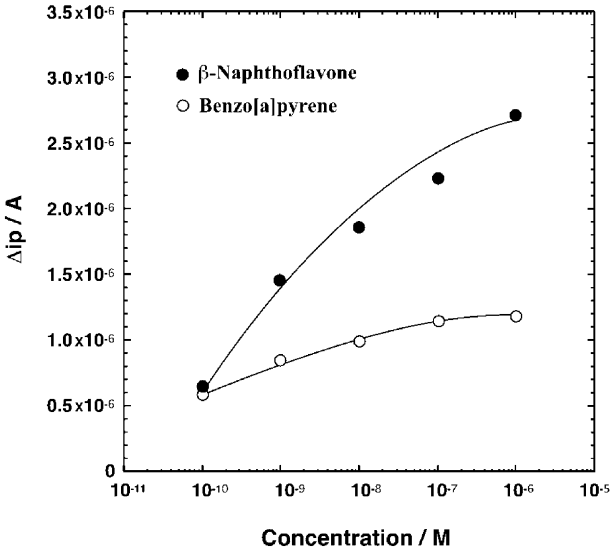

Figure 6. Concentration-dependent changes in the anodic peak current (Δipa) on the Au electrode modified with the $hAhR_{102–446}$. Open circle, β-NF; solid circle, B[*a*]P. All measurements were performed in 10 mM Tris buffer, pH = 7.4, [KCl] = 10 mM, $[Fe(CN)_6]^{4-/3-}$ = 5 mM, at 25 °C.

trode showed good response to β-NF in the wide concentration range from 10^{-10} to 10^{-5} M. The sensitivity of this electrochemical sensing system based on a ligand–receptor interaction was comparable to those of other in vitro screening methods.[11,19,20] B[*a*]P, which has been shown to bind with hAhR, was also detected by the $hAhR_{102–446}$-modified electrode. On the other hand, DMSO used as a control had no significant effect on the sensor under the experimental conditions (data not shown). These results suggest that the $hAhR_{102–446}$ immobilized on the electrode surface maintains its ligand-binding activity. Interestingly, electrochemical responses of the sensor to the PAHs were similar to results from a yeast two-hybrid system, which is typical of in vitro screening methods.[19,20] EC_{50} of B[*a*]P was evaluated 5 nM from Figure 6, this value is comparable to yeast system reported by Miller III.[19] The response of the hAhR-modified bioaffinity sensor may reflect the binding property of the receptor in the cell.

3. Conclusion

The electrochemical detection system reported here showed a good response to the PAHs that have long been known to be hAhR ligands. This system has numerous advantages over currently available AhR-based assays, including increased rapidity and ease of use, low reagent cost, and application for high-throughput screening to characterize the hAhR binding interactions of pharmaceutical, environmental, and industrial chemicals. We therefore envision that this assay will be very suitable for adaptation to any application where cost or high sample numbers are an issue.

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