Densitometry Determination of Dioxins Using Gold Nanoparticle-modified Dioxin Response Element Probes

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Abstract

This study aims to develop a rapid and sensitive densitometry bioassay of dioxins. It is known that dioxin ligand can bind heterodimeric aryl hydrocarbon receptor (AhR) and trigger the formation of the complex of dioxin-AhR, AhR nuclear translocator (Arnt), and dioxin-responsive element (DRE). We have established a rapid and sensitive method using nanoparticle-modified DRE probes based on dioxin-dependent binding of DRE to AhR complex. Dioxins can trigger the binding of nanoparticle-modified DRE probe to AhR complex. The nanoparticle-modified DRE probes in the dioxin-AhR-Arnt-DRE complexes immobilized on the microplates were detected by measuring optical density after silver enhancement. Results showed that as little as 0.01 pM of TCDD could be detected. Dioxins could be quantified accurately with the linear range from 1 nM to 0.01 pM. The coefficient of variation was 5~8% in this bioassay. The optical density of nanoparticle-based silver enhancement was produced in a dose- and time- dependent manner. The dioxin concentrations in fly ash samples

determined by this bioassay were comparable with that by the standard HRGC/HRMS. Thus this bioassay is a sensitive and convenient cell-free bioassay of dioxin that is suitable for screening of dioxins in environmental or food matrices.

Keywords

nanoparticle, dioxin, persistent organic pollutants, aryl hydrocarbon receptor

Abbreviations

AHH, aryl hydrocarbon hydroxylase; AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator; B(a)P, benzo (a)pyrene; CALUX, chemical-activated luciferase gene expression; DRE, dioxin response element; EROD, ethoxyresolufin-O-deethylase; GNP, gold nanoparticle; HPLC, high performance liquid chromatography; HRGC/MS, High-Resolution Gas Chromatography/ Mass Spectrometry; TCDD, 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin; TEQ, Toxic Equivalent Quantity.

Introduction

Detection and quantification of dioxin-like chemicals in biological, environmental and food matrices is of vital importance due to their high toxicity and potential bioaccumulation/biomagnification.^{1, 2} The present regulating procedure has been set to use High-Resolution Gas Chromatography/ High-Resolution Mass Spectrometry (HRGC/ HRMS) systems for dioxin detection.^{3, 4} However, the HRGC/ HRMS measurements are often costly and time-consuming because of its requirements for complicated separation process and expensive instrumentation, which unavoidably decrease the acceptance of HRGC-HRMS in screening large numbers of samples. The chemical-activated luciferase gene expression (CALUX)⁵ and ethoxyresolufin-O-deethylase (EROD) activity bioassay,¹ which have been established on a specific cellular response to dioxins, are both limited for screening purposes due to interference of cellular metabolism with uncertain consequences. In addition, most cell-free bioassays require radioactivity, such as the gel retardation of AhR binding assay (GRAB),⁶ immunoassay using antibody to AhR or dioxin,⁷ which are also unfeasible for extensive screening of samples because of radioactive contamination.

AhR is a ligand-activated transcription factor that mediates much toxicological potency of dioxins. After the binding of dioxin ligand, the cytosolic AhR, which is translocated into the nucleus, undergoes transformation into a high-affinity DRE binding form and then forms a heterodimer with Arnt.⁸⁻¹⁰ The complex of dioxin-AhR-Arnt can recognize DRE and result in transcriptional activation of adjacent genes.¹¹ Hence, the ability of the key biological molecules, such as AhR, Arnt and DREs to recognize a unique structural property of dioxins could be used to establish new detection methods.¹²⁻¹⁴

Table 1*Removal of B(a)P by AHH from fly ash extract*

Before degradation ($\mu\text{g/L}$)	1st ($\mu\text{g/L}$)	2nd ($\mu\text{g/L}$)	3rd ($\mu\text{g/L}$)
3.52	0.02	ND	-
5.56	0.02	ND	-
8.54	0.02	ND	-
9.96	0.02	ND	-
11.26	0.04	ND	-
15.42	0.04	ND	ND
23.78	0.08	ND	ND
31.60	0.14	ND	ND
53.92	0.18	0.02	ND
72.88	0.42	0.02	ND
103.64	0.46	0.02	ND

* ND stands for “not determined”

As a newly rising method of labeling, nanoparticle technology has an overwhelming advantage in the field of bioassay due to its diverse characteristics including non-radioactivity, high electron density, high sensitivity.^{15,16} Niemeyer et al. have done a lot of work on DNA-gold nanoparticle based protein detection. Their researches lay majorly upon DNA-directed immobilization of proteins. The linkage between gold nanoparticle and IgG protein is by hybridization of two complementary oligonucleotides, one of which is functionalized with IgG protein via streptavidin/biotin system.^{17, 18} In our assay, nanoparticle-modified sequence-specific dsDNA has the capability of recognizing the protein complex. It has no need for synthesis of DNA-streptavidin and biotinylation of protein. Biomolecules, such as oligonucleotide probes capped with alkanethiol groups at their ends, are able to attach themselves covalently to nanoparticles.^{19, 20} The application of nanoparticle to probes has provided an access to high sensitivity and ease for detection.

Here, we demonstrate the development and validation of a new AhR-mediated bioassay based on the nanoparticle-modified DRE probes and silver enhancement principle. As shown in Figure 1, when the dioxin triggered the formation of AhR-Arnt complex to acquire high-affinity DRE binding ability, the complexes were transferred and captured onto the surface of microplates via anti-Arnt antibody linkage. The gold nanoparticle-modified DRE (GNP-DRE) probes were subsequently bound to the AhR-Arnt complexes. After silver enhancement, the GNP-DRE probes were detected with optical density and the dioxins could be quantified by densitometry.

Experimental methods

Chemicals, reagents, and oligonucleotide probes

TCDD was purchased from Cambridge Isotope Laboratories,

Inc. (Texas, USA). 15 nm nanoparticles were provided by Sino-American Biotechnology Co. (Luoyang, China). The alkanethiol-modified oligonucleotide sequences of DRE were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. The anti-Arnt (c-19) antibody was purchased from Santa Cruz Biotechnology, Inc. (California, USA). B(a)P and NADPH were all from Sigma (St. Louis, USA).

Preparation of hepatic cytosol containing AhR and Arnt

The AhR and Arnt protein were obtained from Sprague-Dawley rat hepatic cytosol. Treatment of animals was a standard operating procedure according to the humane handling procedure approved by our institutional animal care and use committee. Livers of male rats were perfused with ice-cold physiological saline, and then homogenized at 4°C in a double volume of HEDGK buffer (25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 80 mM KCl). The homogenate was centrifuged at 10,000 g for 20 min and the supernatant was collected after another centrifugation at 105,000 g for 60 min. Then, AhR and Arnt proteins present in the supernatant were stored at -80°C for usage. Protein concentrations were measured by Bradford method using bovine serum albumin as the standard and adjusted to 10–15 mg/mL diluted in HEDGK buffer.

Preparation of gold nanoparticle-modified DRE probes

Seq1. 5'-SH-(CH₂)₆-TACCATTGAGCGGAGTTGCGTGAGAAGAG-3', Seq2. 5'-GACTCTTCTCACGCAACTCCGCTCAATGGTA-3'. The underlined sequences correspond to the AhR/Arnt binding core site and are referred as DRE sequences.^{21, 22} Seq1 (100 μM , 4 μL) dissolved in 46 μL Tris-Cl/EDTA (TE) buffer (pH 8.0) was added to the precipitated gold nanoparticles, which were centrifuged by 14,000 rpm for 20 min. The mixture was stored at 4°C for 12 h, followed by addition of another 50 μL phosphate buffer (0.2 M NaCl, 20 mM Na₂HPO₄/NaH₂PO₄, pH 7.0) and storage at 4°C for another 24 h. The free unlabeled oligonucleotides probes were removed by centrifugation at 4°C, 14,000 rpm for 10 min. Then, the gold nanoparticle-modified Seq1 (GNP-Seq1) sequences were all dissolved in 100 μL phosphate buffer (0.1 M NaCl, 10 mM Na₂HPO₄/NaH₂PO₄, pH 7.0). Finally, GNP-Seq1 was hybridized with Seq2 for 4 h at 25°C in a solution containing 1.5 M NaCl, 50 mM PB and 0.05% SDS to form double-stranded GNP-DRE probe.

Detection of dioxins

The hepatic cytosol was incubated with 2, 3, 7, 8- TCDD at different concentrations (0.01 pM~1 nM, diluted in DMSO) at 20°C for 2 h in dark to form the activated AhR/Arnt complex. DMSO was used as a control. Anti-Arnt (c-19) antibody diluted at 1:100 in carbonate buffer (Na₂CO₃ 0.16 g, NaHCO₃ 0.29 g, 100 mL H₂O, pH 9.6) was immobilized on the microplates through incubation at 4°C overnight. After washing with PBST (0.01 M phosphate buffered, 0.05% Tween20), the activated AhR/Arnt complex in cytosol was added to the microplates

and then incubated at 37°C for 1h to complete the antigen-antibody binding. Then the plates were rinsed with PBST for another 5 min, followed by addition of 100 μ L 0.25% (v/v) fresh acetic anhydride in 100mL 0.1M triethanolamine (pH 8.0) and incubation at 25°C for 10 min. The GNP-DRE diluted at 1:20 with PBS was added to the complex and incubated at 37°C for 1h, and then rinsed with PBST for 3 times and PBN (0.3 M NaNO₃ and 10 mM Na₂HPO₄/NaH₂PO₄ buffer, pH 7.0) for 5 times.

Silver enhancement was carried out by adding 100 μ L of enhancer solution (0.5 g AgNO₃/2mL H₂O, 1.7 g hydroquinone/30 mL H₂O and 2.55 g citric acid/2.35 g trisodium citrate/10 mL H₂O, mixed simultaneously) in each microwell and incubated at 25°C.¹⁵ The enhancement time

was about 1-4 min, and the reaction was stopped by dipping the microplates in double-distilled water for rinse.²³ With the signal amplification, the optical density was recorded in a real-time manner. It is recommended that this solution be used in dark to delay self-nucleation of silver ions, although the enhancer solution could be used in daylight.²⁴ It's worth noting that the microwells must be washed thoroughly with PBN prior to silver enhancement to remove ions (especially chloride ions), which can interfere with silver enhancement process. Furthermore, high temperature (>30°C) and agitation of the microplates during incubation in the enhancer solution can speed up the deposition of silver on the gold surface.

Removal of B(a)P from fly ash

Dioxins in fly ash of burned garbage was extracted with acetone by Soxhlet extraction, purified and enriched by solid phase extraction. In order to remove B(a)P from the samples, it was treated with AHH-containing (aryl hydrocarbon hydroxylase) cytomicrosome and NADPH (50 mM) and then dissolved in cane sugar buffer for incubation with shaking at 28°C for 30 min. After centrifugation at 9000 g for 20 min, the supernatant was collected and analyzed by HPLC/fluorescence to identify the elimination of B(a)P.²⁵ If any B(a)P was detected in the sample, the above procedure was repeated until no B(a)P was detected.

Safety consideration

When dioxins were handled, two pairs of protective gloves with some water between the two layers were worn to avoid penetration of dioxins. After the experiment, the operation platform was exposed to UV light for degradation of dioxins.²⁶

Figure 1

Detection of dioxins based on nanoparticle-modified dioxin response element probes. (GNP-DRE: gold nanoparticle-modified DRE)

Figure 2

Detection of TCDD by nanoparticle-modified DRE probes with silver enhancement. (a) Image of optical density obtained from various concentrations of TCDD in a time-dependent manner. (b) The time-absorbance relationship of various concentrations of TCDD

Results and discussion

Detection of TCDD by nanoparticle-modified DRE probes

After the signal was amplified by silver enhancement, the result could even be observed visually (Figure 2a). Nanoparticle could significantly accelerate the polymerization process of silver particles so that the optical density of nanoparticle-based silver enhancement was produced in a dose- and time-dependent manner (Figure 2b). The signals of optical density were recorded by microplate reader. As the AhR/Arnt complex binding with DRE was induced by dioxins, the amount of AhR/Arnt complex was proportional to the TEQs (Toxic Equivalent Quantity) of dioxins, which were correspondingly related to the nanoparticle-modified DRE probes.

Quantification of dioxin

An optimal time point for terminating the silver enhancement reaction is important, so we explored and finally found that the optical density of silver enhancement at 110 s was correlated linearly with the concentrations of target TCDD. This bioassay based on nanoparticle-modified DRE probes revealed a minimum detection limit of 0.01 pM and showed a linear range of 6 orders from 1nM to 0.01pM with a correlation efficiency ($R^2=0.9611$).

This bioassay based on nanoparticle-modified DRE probes is a hybrid of the AhR-mediated mechanism and the signal amplification of silver-enhanced gold nanoparticle. It eliminates the disadvantage of cell-based bioassay, while keeping the advantage of gold nanoparticle modified probes. The procedure allows effective analysis to be carried out without complicated or expensive instrumentation. This method renders it feasible to proceed with food, drinking water as well as environmental samples.

Removal of B(a)P from fly ash samples by microsomal AHH

As B(a)P is capable of binding to AhR to interfere with the quantification of dioxins in samples, it is necessary to remove B(a)P from the sample before quantification of dioxin. In our assay, B(a)P was removed by AHH, which was obtained from liver cytomicrosome of male Sprague-Dawley rat. Results showed that more than 95% of B(a)P was removed after treatment by AHH at 28°C for 30 min (Table 1). There is no B(a)P detected in the sample after being treated 2-3 times.

Detection of dioxins in fly ash samples

To verify the efficiency of the method for detection of dioxin, we determined the TEQs of fly ash samples by our bioassay. It showed that the detection results of dioxins by nano-DRE and HRGC/MS were comparable with a significant correlation ($R^2=0.9859$) (Figure 3). The TEQs of nano-DRE were a little higher than that by HRGC/HRMS on account of the co-activities of all congeners concerning AhR agonists in the sample.

Figure 3

Comparison of nano-DRE and HRGC/HRMS for detection of dioxins in fly ash

Figure 4

Comparison of dose-response curves for TCDD by CALUX and nano-DRE bioassays. Values represent the mean $\pm$ SD of triplicate independent determinations

Unlike the classical chemical analysis (e.g. HRGC/HRMS), which has limited information on the biological potency, the AhR-based bioassay integrates all activities and potential interactions of all congeners in complex mixture of dioxins. This nanoparticle-based bioassay provides a useful tool in screening dioxins in environmental or food matrices. If the result shows positive, HRGC/MS can be employed to make further analysis to identify the specific compounds. In this way, the expenses of detection for dioxins can be cut down by 80% through eliminating the cost for analyzing negative samples by HRGC/MS.

Comparison of nano-DRE assay with CALUX assay

To compare with CALUX, the dose-response relationship of nano-DRE and CALUX²⁷ for TCDD detection are shown in Figure 4. The sensitivity of nano-DRE was approximately 5 times higher than that of CALUX (EC_{50} of 4×10^{-12} M compared to 2×10^{-11} M, respectively). The low detection limit was 10 times lower (1×10^{-14} M compared to 1×10^{-13} M for the CALUX assay), while it exhibited higher reproducibility (CV of 5–8% compared to 15–30%, respectively). This superiority may be ascribed to the stability of cell-free assay compared to the cell-based assay, which has considerable variation due to variability in cell metabolism.

It is important to eliminate nonspecific signals of nanoparticle, such as signal from binding of antibody with nanoparticle. In order to avoid the nonspecific binding of antibody with nanoparticle, the incubation of nanoparticle-modified DRE with AhR/Arnt in the wells was performed in a relative high pH value (pH 8.5) to keep the antibody in a negatively charged state and posing repulsion with nanoparticle. In addition, the wells were treated with acetic anhydride before the process of DRE probes binding to AhR/Arnt complex, because acetic anhydride could acetylate free amines to neutralize potential positive charge to ensure that the probe will bind specifically to AhR/Arnt complex rather than nonspecifically due to electrostatic interactions.

Conclusions

In conclusion, we have developed a bioassay for dioxin detection that possesses several advantages. Firstly, our bioassay is easy and rapid for dioxin measurement without cell culture, which takes only 5 hours to complete the whole analysis. Secondly, the bioassay is of low cost without the need for complicated instrumentation. All of the materials are commercially available. Thirdly, it can be used for screening of a large number of samples simultaneously. Finally, our method is highly sensitive with a detection limit of 0.01 pM for TCDD and a linear range from 1 nM to 0.01 pM. Therefore, it is a cost-effective and high-throughout method for screening of dioxins.

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