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Biotin–streptavidin enzyme-linked immunosorbent assay for detecting Tetrabromobisphenol A in electronic waste

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ABSTRACT

Tetrabromobisphenol A (TBBPA) is a widely used brominated flame retardant. A sensitive and selective indirect competitive biotin–streptavidin-amplified enzyme-linked immunosorbent assay (BA-ELISA) was developed for detecting TBBPA. The optimal hapten of TBBPA was 2-(2,6-dibromo-4-(2-(3,5-dibromo-4-hydroxyphenly)propan-2-yl)) acetic acid. Several physiochemical factors that influence assay performance, such as optimal coupling concentration of immunogen and antibody, organic solvent, ionic strength, and pH, were studied and optimized. The limit of detection (IC_{10}) was 0.027 ng/mL and the median inhibitory concentration (IC_{50}) was 0.58 ng/mL. The BA-ELISA was highly selective, with low cross-reactivity with TBBPA analogs. Finally, the assay was used to detect TBBPA in electronic waste samples. The results are consistent with those using liquid chromatography, which proves that the proposed immunoassay is accurate and receptive. This BA-ELISA method is suitable for the rapid and sensitive screening of TBBPA in environmental monitoring.

1. Introduction

Tetrabromobisphenol A (TBBPA) is the most widely used brominated flame retardant (BFR), and it mixes with or covalently bonds to host materials. As an additive or reactive flame retardant, TBBPA is commonly used to produce brominated epoxy resins, polycarbonate resins, phenolic resins, unsaturated polyester, high-impact polystyrene, acrylonitrile–butadiene–styrene, and so on. The global consumption of TBBPA has increased from 64,000 t in 1994 to 119,700 t in 2001 [1]. The TBBPA production capacity of China was approximately 18,000 t in 2007 [2]. The worldwide use of TBBPA has transferred this compound from different processes and sources into the environment. Trace concentrations of TBBPA have been detected both in abiotic and biotic media, including indoor air (stationary air samples [3]: $<$3–180 ng/m^3; dust samples [4]: 2300–2900 pg/d), water [5–7] (wastewater [6]: 0.013–0.031 ng/mL), soil [8] (industrial soils: 3.4–32.2 ng/g *dw*; agricultural soil: 0.3 ng/g *dw*), human tissue (serum [9]: $<$1–3.4 pmol/g *lw*; breast milk [10]: 7000 ng/kg *lw*), food (Chinese total diet [11]: $<$LOD–2044 pg/g; eggs [12]: $<$0.1–940 ng/g *lw*) and wild animals (birds of prey [13]: 13 pg/g; dolphin fat [14]: 0.1–418 μg/kg *lw*).

TBBPA is listed in the convention for the protection of the marine environment of the North-East Atlantic as a hazardous substance. TBBPA is classified as an endocrine-disrupting chemical [15] because its molecular structure is similar to that of thyroxine. Other studies have shown that TBBPA is an immunotoxic and neurotoxic compound [16,17]. TBBPA is dehalogenated under anaerobic and aerobic conditions to yield bisphenol A (BPA).

Gas and liquid chromatography techniques have been used to detect TBBPA and its derivatives in different environmental samples [18–22]. However, these instrumental analytical methods are generally expensive, time-consuming, labor-intensive, and require complex pretreatment procedures, which restrict their widespread use for the rapid detection of TBBPA in environmental samples. Compared with instrumental analytical methods, enzyme linked immunosorbent assay (ELISA) is well suited for detecting trace pollutants in the environment because of its high specificity, sensitivity, and throughput. In the literature, ELISA methods have been used to measure polybrominated diphenyl ethers [23–27] and other brominated flame retardants.

This study aims to develop a modified indirect competitive ELISA for TBBPA using a biotin–streptavidin amplification system. Diverse TBBPA haptens, immunogens, and relative polyclonal antibodies were prepared. The established immunoassay procedures were optimized, and then their accuracy and sensitivity were compared with those of the liquid chromatography.

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The biotin–streptavidin-amplified ELISA (BA-ELISA) selectively and sensitively detected TBBPA in various environmental samples and it will be helpful in environmental studies.

2. Materials and method

2.1. Reagents and apparatus

The TBBPA standard and organic materials for hapten synthesis were purchased from J&K Chemical (Beijing, China). Hapten was purified through column chromatography using silica gel (40 μm average particle size) from Shanghai Sanpont Co. Ltd. (China). Bovine serum albumin (BSA), egg albumin (OVA), biotinylated *N*-hydroxysuccinimide ester (BNHS), *N*,*N*-dimethylformamide (DMF), *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), dimethyl sulfoxide (DMSO), hydrogen peroxide, Coomassie Brilliant Blue G250, Tween 20, complete and incomplete Freund's adjuvant, and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sinopharm, China. Streptavidin–horseradish peroxidase (SA–HRP) was purchased from Sangon Biotech (Shanghai, China). All reagents were of analytical grade unless specified otherwise.

The ^{1}H Nuclear Magnetic Resonance (NMR) Spectrometer was an Avance III 400 MHz instrument (Bruker, Switzerland) with $CDCl_3$ solution. Fourier transform infrared spectrometry was performed on a Nicolet 6700 instrument (Thermo, USA). The Multiskan MK3 ELISA reader (Thermo, USA) used to determine absorbance in dual wavelength mode (450 nm/650 nm), with polystyrene 96-well microplates, was purchased from Sangon Biotech. TBBPA–protein conjugate was characterized on a UV-2012 PC spectrophotometer (UNICO, USA). Ultrapure water used was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

HPLC analysis was performed on an Agilent LC1100 HPLC system equipped with an Agilent Eclipse Plus C18 (250 mm × 4.6 mm, 5 μm). The mobile phase consisted of methanol and water in the ratio 80:20, v/v at a flow rate of 0.3 mL/min. The injection volume was 20 μL and detection was at 280 nm.

2.2. Buffers and solutions

Phosphate-buffered saline (PBS: NaCl 137 mmol/L, KCl 2.7 mmol/L, Na_2HPO_4 10 mmol/L, KH_2PO_4 2 mmol/L), carbonate buffer solution (CBS: 15 mmol/L Na_2CO_3, 34.9 mmol/L $NaHCO_3$), PBST (PBS with 0.05% Tween 20), phosphate–citrate buffer (0.1 mol/L citric acid, 0.2 mol/LNa_2HPO_4; pH=4.3), and TMB substrate solution (0.4 mL, 2.5 g/L TMB ethanol solution, 10 mL phosphate–citrate buffer, 10 μL 30% H_2O_2) were used.

2.3. Synthesis of TBBPA hapten

TBBPA molecules do not contain functional groups that can connect with proteins directly. Therefore, TBBPA hapten must be synthesized first. The synthesis reactions are illustrated in Fig. 1. The results of the hapten synthesis and characterization are given below.

Bromoethanoic acid (0.5109 g, 3.677 mmol) dissolved in 5 mL of DMF was added dropwise to a mixture of TBBPA (2 g, 3.677 mmol) and NaOH (0.294 g, 7.354 mmol) in 10 mL of DMF with stirring. The mixture was allowed to react isothermally for 6 h at 80 °C, and the mixture was cooled to ambient temperature. Then, 30 mL of water was added to the reaction mixture and acidified to pH 3 using a HCl solution. The precipitates were obtained and extracted with ethyl acetate (20 mL × 3). The organic phase was washed with water and dried with anhydrous sodium sulfate. After evaporation under vacuum, the light yellow residue was purified via silica column chromatography (*n*-hexane:acetone=4:1) and identified as 2-(2,6-dibromo-4-(2-(3,5-dibromo-4-hydroxyphenly)propan-2-yl)) acetic acid, $C_{17}H_{14}Br_4O_4$, m.w.: 601.92, yield: 71.5%, and m.p.:167–170 °C.

IR (KBr) ν (cm^{-1}): 3475.69 (O–H stretching vibration), 1733.86 (C–O stretching vibration), 1577.22 and 1472.99 (C–C framework vibration), 875.55, 778.72, and 734.95 (C–H flexural vibrations), and 621.08 (C–Br stretching vibration). ^{1}H NMR ($CDCl_3$) δ (ppm): 11.5 (1H, COOH), 7.31 (2H Aromatic H), 4.19 (2H–OCH_2COOH), 2.50 (1H, –OH), and 1.60 (6H, $2CH_3$).

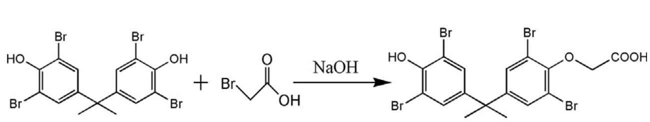

Fig. 1. Synthesis route of TBBPA hapten.

2.4. Preparation of immunogen and coating antigen

A contact portion between hapten and carrier protein is called linking arm [28], which could become an antigenic determinant and specifically conjugate with antibody. In order to reduce the specific binding caused by linking arm, different coupling methods are often used for preparing immunogen and coating antigen. So in this study, the activated ester method and the mixed anhydride method were employed to couple hapten to the carrier proteins using the terminal carboxyl group on a TBBPA hapten molecule. The details were as follows: for the activated ester method, equimolar TBBPA hapten, NHS and DCC were dissolved in 1 mL DMF and magnetically stirred for 8 h at 4 °C. The obtained supernate was added dropwise into BSA solution (12 mg/mL, in PBS) and then stirred at 4 °C overnight. After complete reaction, the suspension was dialyzed against PBS (0.01 M, pH 7.4) for 3 d. For the mixed anhydride method, 300 mg (0.5 mmol) of TBBPA hapten was dissolved in 1 mL DMF. Equimolar isobutylamine and *n*-butyl chloroformate ester were sequentially added and magnetically stirred for 1 h at 4 °C. After reaction, 12 mg/mL of OVA suspension was added dropwise and was allowed to react for 5 h at 4 °C. After complete reaction, the suspension was dialyzed against PBS for 3 d. The prepared hapten–protein conjugates BSA–TBBPA and OVA–TBBPA were used as the immunogen and the coating antigen, respectively.

All protein conjugates were identified using a UV–vis spectrophotometer and the coupling ratios were estimated based on mole absorbance ε and calculated using the following equation [29,30]:

$$\textit{coupling ratio} = \frac{\varepsilon_{\text{conjugate}} - \varepsilon_{\text{protein}}}{\varepsilon_{\text{hapten}}} = \frac{(\text{OD}_{\text{conjugate}} - \text{OD}_{\text{protein}})\, C_{\text{hapten}} M_{\text{protein}}}{\text{OD}_{\text{hapten}}\, M_{\text{hapten}}\, C_{\text{protein}}} \tag{1}$$

2.5. Preparation of biotinylated antibody

Rabbit polyclonal anti-TBBPA antibodies (pAb-TBBPA) were prepared as described in previous reports of our research team [31]. After immunization with TBBPA–BSA for 4 months, the polyclonal antibodies were separated and purified from rabbit serum. Biotinylated TBBPA antibodies (Bi-pAb-TBBPA) were prepared as follows: 5.0 mg of purified pAb-TBBPA was dissolved in 0.1 mol/L sodium carbonate buffer (pH 9.6) at concentrations of 1.0–2.0 mg/mL. The antibody solution was mixed with 1.0 mg/mL BNHS in DMSO in the mass ratio of 1:10. The mixture was stirred for 4 h and then dialyzed against PBS for 3 d. The as-obtained biotinylated antibodies were stored at 4 °C before use.