Development of a noncompetitive phage anti-immunocomplex assay for brominated diphenyl ether 47

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A B S T R A C T

We present a new application of the noncompetitive phage anti-immunocomplex assay (PHAIA) by converting an existing competitive assay to a versatile noncompetitive sandwich-type format using immunocomplex binding phage-borne peptides to detect the brominated flame retardant, brominated diphenyl ether 47 (BDE 47). Three phage-displayed 9-mer disulfide-constrained peptides that recognize the BDE 47–polyclonal antibody immunocomplex were isolated. The resulting PHAIAs showed variable sensitivities, and the most sensitive peptide had a dose–response curve with an SC50 (concentration of analyte producing 50% saturation of the signal) of 0.7 ng/ml BDE 47 and a linear range of 0.3–2 ng/ml, which was nearly identical to the best heterologous competitive format (IC50 of 1.8 ng/ml, linear range of 0.4–8.5 ng/ml). However, the PHAIA was 1400-fold better than homologous competitive assay. The validation of the PHAIA with extracts of house furniture foam as well as human and calf sera spiked with BDE 47 showed overall recovery of 80–113%. The PHAIA was adapted to a dipstick format (limit of detection of 3.0 ng/ml), and a blind test with six random extracts of local house furniture foams showed that the results of the PHAIA and dipstick assay were consistent, giving the same positive and negative detection.

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Antibody-driven specificity and affinity have made immunoassays widely accepted analytical tools for the detection of a variety of substances, including small-molecular-weight analytes such as environmental contaminants, pesticides, pharmaceuticals, personal care products, toxins, and hormones [1–3]. Immunoassays are generally categorized into one of two functional formats; noncompetitive sandwich type or competitive. Macromolecules with two or more nonoverlapping epitopes can be detected by a noncompetitive immunoassay in which one antibody immobilized on the solid support captures the target molecule and a secondary antibody conjugated with signal-producing molecules detects the captured protein. In the case of small analytes, most of the antigen is buried in the antibody binding pocket after binding; therefore, the analyte cannot be simultaneously recognized by a second antibody. For that reason, the competitive format has been the method of choice for small molecule analytes.

The use of an antibody capable of recognizing an analyte-bound antibody enhances the affinity and specificity of the primary antibody because of the formation of a ternary complex, which translates into an improved noncompetitive assay with enhanced sensitivity [4–8]. Although there have been efforts to produce these anti-immune complex antibodies by immunization with analyte–antibody complexes, the method has rarely been successful. In addition, polyclonal antibodies (PAbs) cannot be used as an immunogens for the anti-immune complex antibody because of their heterogeneous nature. An alternative method, called open sandwich assay, was recently introduced for the development of homogeneous noncompetitive assays for small analytes [9–12], but the method relies on the use of recombinant antibody fragments that must show a markedly different association of the light and heavy chains in the presence or absence of the antibody, making it case specific.

To circumvent those limitations, we recently introduced the phage anti-immunocomplex assay (PHAIA) technology for the development of noncompetitive assays for small analytes [13,14]. Briefly, a phage-displayed library is selected (panned) using the analyte–antibody immunocomplex as selector molecule, and the phage-borne...
peptides that are specific for the immunocomplex, but bind inefficiently to the free antibody, are chosen. These phage clones are used as secondary reagents in the development of the immunoenasay, and the signal is generated with an anti-phage antibody coupled to horseradish peroxidase (HRP). The assay showed significantly enhanced sensitivity compared with a hapten-based competitive assay. In addition, PHAIA is particularly useful in the case of PAb-based assays because it can be a viable alternative to a heterologous competitive assay that involves the synthesis of structural variants of the immunizing hapten to minimize the cross-reactivity of immunoglobulin Gs (IgGs) to the competing hapten [15-17]. Moreover, the heterogeneous nature of PAbs does not allow them to be used as immunogens to isolate anti-immunocomplex antibodies.

Although several anti-immunocomplex phage peptides have been selected for monoclonal antibodies (MAbs) [13,18,19], the method has been applied only to one PAb [14]. To explore the possibility of further expanding the scope of PHAIA to PAb-based assays, we developed a PHAIA using a PAb for a common congener of brominated flame retardants, brominated diphenyl ether 47 (BDE 47). BDEs have been used intensively as flame retardants in a variety of consumer products, including plastics, textiles, furniture, and electronic devices, to reduce the risk of fire. Concerns have risen regarding the possible dispersion of those compounds in the environment and their effects on human health through exposure by dietary intake, breathing, or direct contact [20-22]. BDEs have been found in a variety of environmental matrices [23,24]. Although instrumental analysis of BDEs, such as gas chromatography (GC) and GC-mass spectrometry (GC-MS), give reliable and accurate detection [25], such methods might not be suitable for rapid monitoring of a great number of samples. Recently, we reported the development of the PAb-based enzyme-linked immunosorbent assay (ELISA) for BDE 47 for screening human exposure and environmental contamination [26]. The goal of the current study is to extend the scope of our PHAIA technology by developing the PAb-based PHAIA for BDE 47 that provides higher potential application to various detection systems. For this study, we used the protein-A-purified PAb isolated for the BDE 47 ELISA. This article describes the selection of the BDE 47-Pab immunocomplex binding peptide, development of a sensitive non-competitive two-site sandwich assay, validation of the two assays using the extracts of house furniture foams and human and calf sera, and adaptation to a simple dipstick format.

Materials and methods

Materials

All reagents were of analytical grade unless otherwise specified. The synthesis of hapten and production of antiserum for BDE 47 were reported previously [26]. BDE 47, BDE congeners, and metabolites were purchased from AccuStandard (New Haven, CT, USA). Mouse anti-M13 MAb–HRP and PD-10 desalting columns were purchased from GE Healthcare (Piscataway, NJ, USA) and helper phage M13KO7 was purchased from New England Biolabs (Ipswich, MA, USA). A BCA Protein Assay Kit for the quantification of purified antibody, a protein A affinity column for IgG purification, and 3,3’,4,4’,5,5’-hexamethylbenzidine were purchased from Pierce (Rockford, IL, USA). Bovine serum albumin (BSA), polyethylene glycol 8000 (PEG 8000), Tween 20, 3,3,5,5’-tetramethylbenzidine (TMB), and nickel chloride were obtained from Sigma (St. Louis, MO, USA).

Phage-displayed peptide libraries and biopanning

A random phage-displayed peptide library with an estimated diversity of 3 x 10^8 independent clones was constructed on the phagemid vector p8V2 [27]. This is a dsDNA-constrained library expressing peptides of 7–11 amino acid residues flanked by two cysteine residues and linked to the N terminus of the major pVIII phage coat protein through a long glycine-rich spacer [GGG-(X)4-30-2(GGGG)], for the selection procedure, 6 wells of two Nunc–Immuno plates were coated with protein-A-purified BDE 47 PAb (10 μg/ml) in 100 μl of phosphate-buffered saline (PBS) for 1 h at 37 °C. After a blocking for a 1-h incubation at 37 °C with 3% BSA in PBS, 150 μl of a phage library in BPBSM (1% BSA in PBS containing 5% methanol [MeOH]) was added to the wells of the one plate. BPBSM (100 μl) containing 100 ng/ml BDE 47 was added to the wells of the other plate. The plates were incubated for 1 h at room temperature with gentle agitation using an orbital plate shaker. The unbound phage peptides were transferred into the wells of the plate that had been incubated with 100 ng/ml BDE 47 and washed with PBST (PBS containing 0.05% Tween 20). The plate was incubated for 2 h at 4 °C with gentle shaking. The plate was washed 10 times with cold PBST and shaken for 30 min at 4 °C with wells filled with PBST. This procedure was repeated once more. Bound phages were eluted by 10-min incubation with 100 μl of 0.1 M glycine-HCl (pH 2.2) per well and neutralized with 5.7 μl of 2 M Tris (pH unadjusted). Six hundred microliter of concentrated log-phage Escherichia coli ARI 292 cells (Affymax Research Institute, Palo Alto, CA, USA) was infected with equal volume of phage eluate by 30-min incubation at 37 °C and then grown in 10 ml SOP medium (Luria–Bertani [LB] medium containing 0.25% K2HPO4, 0.1% MgSO4, 0.1% glucose, and 100 μg/ml ampicillin) to an OD600 of 0.4. M13K07 helper phage at a multiplicity of infection of 10:1 was added. After a period of 30 min at 37 °C without shaking, arabino- and kanamycin were added to final concentrations of 0.02% and 40 μg/ml, respectively, and the cultures were incubated overnight at 37 °C with vigorous shaking. Phage from liquid cultures was obtained by clearing the supernatants by centrifugation at 12,000g for 15 min, precipitated by adding 0.2 volumes of PEG–NaCl solution (20% PEG 8000 in 2.5 M NaCl) on ice for 1 h, and centrifuged as above. Phage pellets were resuspended in 2 ml of sterile PBS and titrated in ARI 292. A number of 10^10 transducing units were used for the next round of selection. The second and third rounds of panning were carried out using the sample procedure described above except that the amounts of antibody coated in the second plates, which were used for panning after counter selection of phages in the first plate, were gradually reduced to 5 and 1 μg/ml for the second and third rounds of panning, respectively. After three rounds of panning, serial dilutions of individual amplified phage clones were tested for their ability to bind to the BDE 47–antibody immunocomplex by a phage ELISA. Positive clones were further selected by checkerboard titration and submitted for DNA sequencing using the primer ON891 (5′ggagcttgcaggggtc) (Division of Biological Sciences, Automated DNA Sequencing Facility, University of California, Davis).

Screening of phage eluate for positive clones by phage ELISA

After three rounds of panning, microcentrifuge tubes containing 1 ml of ARI 292 cell culture in 20 ml of LB (OD600 = 0.5 AU) were centrifuged at 5000 rpm for 5 min and then 900 μl of medium was removed. Cell pellets were resuspended with 10 μl of diluted phage eluates, and the tubes were incubated for 20 min at 37 °C. The infected cells were plated on LB agar ampicillin and grown overnight. A total of 17 individual clones were picked and used for inoculation of tubes containing 5 ml of SOP, and cells were grown with vigorous shaking at 37 °C. After cultures reached an OD600 of 0.4 AU, 10 μl of M13K07 helper phage at a concentration of 1 x 10^11 transducing units/ml was added. Cultures were then incubated for 30 min at 37 °C without shaking to allow
infection of the cells. Arabinose and kanamycin were added as described above, and cultures were grown overnight with shaking at 37 °C. The next day, the cells were pelleted by centrifugation at 10,000 rpm for 5 min and the supernatants were used for screening. Phage ELISA for screening the phages that react with the BDE 47–antibody complex was performed by the direct addition of 10 μl of supernatants to wells coated with 1 μg/well protein-A-purified BDE 47 PAb with or without the addition of 25 μl of 200 ng/ml BDE 47 per well. PBST was added to the final volume of 100 μl/well.

Preparation of concentrated phage peptide

Phage clones showing different signals in the presence or absence of BDE 47 were selected, and individually amplified phage clones were obtained as described above. After two steps of precipitation with PEG 8000–NaCl, the phage particles were suspended with 5 ml of PBS that was supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN, USA) and sodium azide at 0.05%. The preparation was filtered (0.45 μm) and stored in aliquots at 4 and –80 °C.

PHAIA

ELISA plates were coated with protein-A-purified BDE 47 PAb at a concentration of 10 μg/ml in PBS by incubation for 1 h at 37 °C. The amount of PAb for coating and the dilutions of phage peptide were determined by the checkerboard titration method. The assay was performed on plates coated with the optimized amount of antibody and blocked for 1 h at 37 °C with 3% skim milk in PBS. Next, 50 μl of serial dilutions of BDE 47 standard in PBS containing 40% MeOH (PBSM) was mixed with a 1:4000 dilution of peptide (6.3 × 10^9 particles/ml) prepared in PBST, and then 100 μl of the mixture was added to the wells of the plate followed by incubation for 1 h at room temperature. The plate was washed 10 times with PBST, and 100 μl of anti-M13 phage MAb conjugated with HRP (1:5000 dilution in PBST) was added. After a 1-h incubation and washing, the peroxidase activity was developed by adding 100 μl of peroxidase substrate (25 ml of 0.1 M citrate acetate buffer [pH 5.5], 0.4 ml of 6 mg/ml dimethyl sulfoxide [DMSO] solution of TMB, and 0.1 ml of 1% H2O2), which was dispensed into each well. The enzymatic reaction was stopped after 15–20 min by the addition of 50 μl of 2 M H2SO4, and the absorbance at 450 nm (corrected at 600 nm) was read in a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

Cross-reactivity

The specificity of the noncompetitive assay was characterized by determining its cross-reactivity with structurally related BDE congeners in the range of 0–100 ng/ml. Data were normalized, and the compound concentration corresponding to the midpoint of the curve (which corresponds to the concentration of analyte producing 50% saturation of the signal [SC50]) was used to express the cross-reactivity of the assay according to the following equation: % cross-reactivity = 100 × [SC50 (BDE 47)]/SC50 (cross-reacting compound).

Solvent effects

DMSO and MeOH were added to PBS to final concentrations of 0%, 10%, 20%, and 40%. For assay, an adequate amount of solvent solution with serial dilutions of BDE 47 standard was mixed with an equal volume of the phage peptides diluted in PBST. The rest of the procedures are the same as those described above.

Matrix effect and assay validation

Calf serum was diluted with PBSM to final concentrations of 0%, 5%, 10%, and 20%. To produce the dose–response curves, serial dilutions of BDE 47 standard prepared with each concentration of serum solutions were mixed with an equal volume of phage peptide solution diluted in PBST. The assay procedures are the same as those described above. The slope and maximal signals of the dose–response curves were compared to evaluate matrix effects. The PHAIA and dipstick format were validated by performing the recovery test and blind test. House furniture foams with no BDEs were used as the control sample for the recovery test. The sample extracts were spiked with BDE 47 standard. The calf and human sera were spiked with BDE 47, and the samples were diluted with the assay buffer containing control calf serum at 5%. The extracts of house furniture foams collected from local houses were used for the blind test. The sample extracts were prepared following the method established in this laboratory [26].

Dipstick assay

For the dipstick assay, 2 μl of serial dilutions of protein-A-purified 1309 PAb in PBS was spotted onto a 0.45-μm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). After drying (~ 40–60 min) at room temperature, the membrane was blocked by soaking in 5% skimmed milk in PBS for 40 min. After rinsing in PBST for 5 min, strips were cut, dried, and kept at 4 °C until used. The assay was performed by dipping the membrane strips for 30 min in PBS mixed with an equal volume of PBSTM spiked with BDE 47 or sample extracts from house furniture foams. The strips were washed with PBST for 5 min by gentle agitation, and then the strips were incubated with anti-M13 MAb–HRP for 10 min (1:4000 dilution). After washing, the color of the HRP activity was developed using the diaminobenzidine–nickel chloride substrate mix.

Results

Biopanning

For Fig. 1A is a schematic diagram of the PHAIA. The method requires the selective reaction of the phage-borne peptide with the immunocomplex giving minimal to no reactivity with the unbound antibody. Due to the small size of the peptide and the stringency imposed during the selection process, this condition can be more easily accomplished than with the use of anti-immunocomplex antibodies. Biopanning for the selection of anti-immunocomplex phage-borne peptides was carried out employing the mixture of phage-displayed peptide libraries with an insertion of 7–11 random amino acid sequences flanked by cysteine residues that display circular peptide loops on the major coat protein of phage particles. To obtain high-affinity phage peptides, we used stringent conditions during the panning procedure. Phage peptide libraries were first counter selected by incubation in the wells coated with BDE 47 PAb in the absence of analyte. Unbound phage peptides were then transferred to other wells that had been incubated with 10 μg/ml analyte for 1 h at room temperature and washed. As the panning proceeded, the amount of antibody for the selection was gradually decreased to remove weak binders by increased competition. Conversely, the amount of antibody for counter selection remained constant to remove nonspecific binders. In the last panning step, BDE 47 was added to the phage library after counter selection at a concentration of 10 ng/ml to competitively remove weak binders. A total of 17 phage clones were
selected for screening by phage ELISA (Fig. 1B) in the presence (50 ng/ml) or absence of BDE 47. All tested phage clones reacted with the immunocomplex, showing negligible cross-reactivity with the unbound antibody. The sequences of these clones are presented in Table 1. Only three different sequences (designated as C2, C2-1, and C2-2) were identified, and all of them corresponded to 9-mer peptides despite the fact that the libraries used expressed peptides in the 7- to 11-mer range. Sequence C2-1 occurred in most of the clones (11 of 17 tested clones, Fig. 1), and all clones had the consensus sequence RDTXXE, indicating that the shared amino acids may be important for specific binding to the immunocomplex.

**Phage peptide-based noncompetitive assay**

Representative clones for each of the three sequences were amplified and semipurified by double precipitation as described above. Serial dilutions of phage particles were added to the wells of the plate coated with four different concentrations of protein-A-purified antibody (10, 5, 2.5, and 1.25 μg/ml) in the absence or presence of BDE 47 (0 or 20 ng/ml). Fig. 2 presents the result with clone C2-1. Similar results were observed for the other two clones (data not shown). The maximal signal difference was observed at 10 μg/ml antibody coating with 6.2 × 10^8/ml phage particles. With a higher number of phage peptides, the precipitation of enzymatic products was observed increasing background signal. The signal difference gradually decreased as the amount of antibody coating decreased. The assay sensitivities for each of the three clones were estimated using the conditions determined by the above method. The dose–response curves are presented in Fig. 3. The assay setup with clone C2-1 performed with the highest sensitivity (SC50 = 6.4 ng/ml), followed by clones C2-2 and C2 (SC50 values of 14.2 and 22.3 ng/ml, respectively).

**Solvent effects**

The addition of solvents to the assay buffer can sometimes enhance the assay performance when the analyte is highly lipophilic. In addition, a high tolerance to solvents may allow the analysis of unknown samples without excessive dilution of the sample extract decreasing the limit of quantification. For these reasons, we tested the effect of two common solvents, MeOH and DMSO, on assay performance. The assay was carried out using PBS containing five different concentrations of solvents (0%, 5%, 10%, 20%, and 40%) (Fig. 4). For the MeOH, there was no significant change in maximal signals over the concentrations. However, assay sensitivity gradually improved as the concentration of MeOH increased. An SC50 value of approximately 1 ng/ml was observed at 20% MeOH, a 6-fold improvement compared with the SC50 value at 0% MeOH. A similar result was obtained with DMSO up to a concentration of 10%, but at 20% there was a 40% decrease in maximal signal, indicating that MeOH is the first choice for the preparation of the assay buffer. Dose-dependent curves were not obtained at 40% MeOH and DMSO, showing near-background signals (data not shown). Thus, we used the assay buffer containing a final 20% MeOH throughout this study.

**Dose–response curve at optimized condition**

Fig. 5 shows a representative PHAIA dose–response curve generated with optimized conditions and the inhibition curve of the homologous competitive ELISA. The SC50 value and linear range

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**Table 1** Peptide sequences isolated with the PBDE PAb immunocomplex.

<table>
<thead>
<tr>
<th>Clone name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2</td>
<td>CFGRDITFEVC (4)</td>
</tr>
<tr>
<td>C2-1</td>
<td>CVHRDITYEY (11)</td>
</tr>
<tr>
<td>C2-2</td>
<td>CVGRDTVHEFC (2)</td>
</tr>
</tbody>
</table>

*Note.* A total of 17 clones were sequenced. The numbers of isolates bearing the same sequence are indicated in parentheses.
of the PHAIA were 0.7 and 0.3 to 2 ng/ml BDE 47, respectively. The linear range was defined by the BDE 47 concentration, causing 10% and 90% saturated binding of phage peptide. The IC50 value of the homologous ELISA was 1000 ng/ml, showing uncompleted inhibition even at 5000 ng/ml BDE 47. As compared with the IC50 of homologous ELISA, the PHAIA showed 1400-fold improved assay sensitivity. Compared with the sensitivity of heterologous ELISA, the PHAIA had the same limit of detection (LOD) but a 4-fold narrower linear range. However, the poly-binding nature of phage particles makes the sensitivity of PHAIA tunable by adjusting the amount of phage peptide or anti-M13 phage antibody–HRP. For example, the use of lower amounts of phage peptide or anti-M13 phage antibody–HRP shifted the slope of the curve to the right and the extended linear range with a loss of sensitivity (data not shown). The standard curve in Fig. 5 was the most sensitive PHAIA, with the highest allowable number of phage particles resulting in a relatively narrow linear range. These conditions also result in the greatest assay precision.

Cross-reactivity

The specificity of the PHAIA was evaluated by the cross-reactivity test with several common BDE congeners by generating dose–response curves for each of them (Table 2). The highest concentration of tested compounds was 1000 ng/ml. As shown in Table 2, 2% and 5% cross-reactivity was observed for BDE 49 and 99, respectively, but for other compounds the cross-reactivity was negligible, showing less than 0.24%, in agreement with what has been observed for the chemical hapten-based competitive assay [26].

Matrix effect and PHAIA validation

The excellent sensitivity and specificity of an immunoassay makes sample preparation simple. Particularly for liquid samples such as serum and urine, the dilution of samples with an assay buffer is generally a preferred sample preparation method for an
Fig. 4. Effect of MeOH (A) and DMSO (B). PBST containing 12 × 10^6 phage peptide was mixed with the same volume of PBS containing different concentrations of each solvent and BDE 47, and 100 μl of the mixture was added to each well coated with 1.0 μg of BDE antibody. Then 100 μl of anti-phage MAb–HRP solution in PBST (1:4000 dilution) was added and incubated for 1 h. Each value represents the mean value of four replicates.

Fig. 5. Representative dose–response curve. Serial dilutions of BDE 47 in PBS containing 40% MeOH were mixed with 6 × 10^8 particles/ml diluted in PBST. Then 100 μl of the mixture was added to the antibody-coated wells. For homologous competitive ELISA, the serial dilutions of BDE 47 in the same assay buffer were mixed with an equal volume of BDE 47 PAb diluted in PBST, and 100 μl was added into the each well of the plate coated with immunizing hapten. Each point represents the mean value of four replicates.

Adaptation of PHAIA to dipstick format

The generation of positive signals by a noncompetitive sandwich-type assay makes the dipstick assay very useful when the assay is needed to be adapted to an on-site detection system, enabling one to easily distinguish the color intensity at a low concentration from that at zero concentration. We developed the dipstick assay by immobilizing immunoaffinity-purified 1309 PAb on the nitrocellulose strips. Fig. 7A shows an evaluation of the sensitivity of the dipstick format. The LOD by this format is approximately 3.0 ng/ml BDE 47 because the intensity of colors at this concentration is clearly distinguishable from that by nonspecific binding at zero concentration. Fig. 7B shows a recovery test using the extracts of house furniture foams spiked with BDE 47 standard (0%, 0.1%, 0.5%, 1%, and 3%). All concentrations of spiked BDE 47 were detected by the development of visible colors, showing no nonspecific colors developed at zero concentration. The assays were further validated by performing a blind test using six extracts of house furniture foams collected from local houses. Table 4 shows the concentrations detected by the PHAIA and dipstick formats. The strip immobilized with antibody without adding analyte to estimate background signal because the diaminobenzidine–nickel chloride substrate mix can generate background signals by prolonged incubation.
Table 2
Cross-reactivity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IUPAC</th>
<th>SC&lt;sub&gt;90&lt;/sub&gt; (ng/ml)</th>
<th>Cross-reactivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDE 47</td>
<td>2,2',4,4'-Tetrabromodiphenyl ether</td>
<td>0.7</td>
<td>100</td>
</tr>
<tr>
<td>BDE 15</td>
<td>4,4'-Dibromodiphenyl ether</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.24</td>
</tr>
<tr>
<td>BDE 49</td>
<td>2,2',4,5'-Tetrabromodiphenyl ether</td>
<td>35</td>
<td>2</td>
</tr>
<tr>
<td>BDE 99</td>
<td>2,2',4,4,5-Pentabromodiphenyl ether</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>BDE 100</td>
<td>2,2',4,4,6-Pentabromodiphenyl ether</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.24</td>
</tr>
<tr>
<td>BDE 153</td>
<td>2,2',4,5,5'-Hexabromodiphenyl ether</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.24</td>
</tr>
<tr>
<td>BDE 154</td>
<td>2,2',4,5,6,6'-Decabromodiphenyl ether</td>
<td>&gt;1000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.24</td>
</tr>
<tr>
<td>Phenyl ether</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Phage peptide binding was not observed up to 1000 ng/ml of compounds.

Conclusion

We have described the development of a sensitive phage-borne, peptide-based, two-site, sandwich-type noncompetitive assay for the detection of BDE 47, a common flame retardant. There is great concern over its increasing environmental presence. The presented phage-peptide-based technology can be a general method for an easy conversion of existing competitive assays to versatile noncompetitive formats for small molecules to meet the demanding needs in developing high-throughput screening methods providing significantly improved sensitivity and high adaptability to various detection systems. The main advantageous features of the technology described in this study are as follows. First, the general procedures and time for PHAIA development can be shortened by using immunoaffinity-purified PAbs. In our previous PAB-based PHAIA, we isolated analyte-specific PAbs using a hapten affinity column for which we covalently immobilized immunizing hapten–protein conjugate onto the beads packed in a column. In this study, we demonstrated that the PAbs obtained by using a commercially available immunoaffinity column (protein A immunomofinity column) can be a good source for antibodies used in PHAIAs, eliminating the need for constructing the hapten affinity column for each antiserum. Second, the technology can be universally applied to all types of compounds. We have reported the PHAIAs for hydrophilic and moderately hydrophilic compounds. In this study, we showed that the PHAIA can be developed against highly lipophilic compounds. The solvent tolerance of the PHAIA was similar to that of the competitive ELISA, indicating that the solvent effect on the PHAIA appears to be dependent on the tolerance of the primary antibody. Third, the technology can be an alternative to an approach for a chemical hapten-based heterologous assay. The extensive chemical synthesis of structural variants of immunizing hapten is frequently required for PAB-based competitive assays to improve assay sensitivity. Ahn and coworkers developed a sensitive heterologous competitive assay for BDE 47 by a synthesis of several derivatives of the immunizing hapten and subsequent time-consuming evaluation [26]. Although this approach has been commonly accepted in chemical synthesis-based laboratories, the method is not an easy task in most other laboratories. Using the huge diversity of a phage peptide library as a heterologous binding source, we successfully developed versatile noncompetitive formats for small molecules and amino acids. Application of the PHAIA in a simple assay format such as immunochromatography can reduce LOD values, enhancing accuracy of on-site rapid detection and not requiring an expensive instrument for signal readout.

Interestingly, the PHAIA sensitivity is improved 6-fold in the presence of 20% MeOH in the assay buffer compared with the
sensitivity at 0% MeOH, and this may help to process less diluted sample extracts decreasing the limit of quantification. The PHAIA and dipstick formats were validated performing the recovery test using the human and calf sera, the spiked extracts of house furniture foams, and the blind test employing six concentrations of spiked BDE 47 showing colors developed from background signals. The dipstick assay detected approximately 3.0 ng/ml BDE 47 in assay buffer, providing distinguishable colors from background signals. The LOD of the dipstick assay was approximately 3.0 ng/ml BDE 47 containing 7 × 10⁶ phage peptide was mixed with the same volume of PBS containing 40% MeOH and BDE 47 standard or sample extracts. (A) Evaluation of the sensitivity of the dipstick assay. (B) Validation of the dipstick assay with the extracts of house furniture foams spiked with BDE 47 at 0%, 0.1%, 0.5%, 1%, and 3% (w/w).

Table 4

<table>
<thead>
<tr>
<th>Sample</th>
<th>Detected by PHAIA (%)</th>
<th>Dipstick assay¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>0.98</td>
<td></td>
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<td>V</td>
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<tr>
<td>VI</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Note: ND, not detected.

¹ Protein-A-purified BDE 47 PAb was immobilized on nitrocellulose membrane at the amounts of 1.0, 0.5, and 0.3 µg per spot from left. The assay methods are as described in Materials and Methods.

Acknowledgments

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References


