A camelid VHH-based fluorescence polarization immunoassay for the detection of tetrabromobisphenol A in water

Kai Wang, Zhiping Liu, Pei Ji, Jianfeng Liu, Sergei A. Eremin, Qing X. Li, Ji Li and Ting Xu*

Tetrabromobisphenol A (TBBPA) is a widely used flame retardant and is classified as an endocrine disruptor. A convenient and sensitive fluorescence polarization immunoassay (FPIA) for TBBPA was developed with a camelid variable domain of heavy chain antibody (VHH) being specific for TBBPA. 2,2-bis(3,5-dibromo-4-hydroxyphenyl)-propane (hapten T1) was conjugated with ethylenediamine fluorescein thiocarbamoyl (EDF) to form a tracer that binds with anti-TBBPA VHH. The limit of detection and half-maximum inhibition concentration of TBBPA by FPIA were 5 ng mL$^{-1}$ and 90 ng mL$^{-1}$, respectively. This FPIA was used to detect TBBPA in water samples without any pretreatment and each analysis was performed within 20–30 min. The recovery of TBBPA from spiked water samples ranged from 73% to 102%, with coefficients of variation ranging from 7% to 14%. The FPIA exhibited high potential for rapid and accurate determination of TBBPA in real water samples.

1. Introduction

Tetrabromobisphenol A (2,2-bis(3,5-dibromo-4-hydroxyphenyl)-propane; TBBPA) is one of the ubiquitous brominated flame retardants (BFRs), which are used in various industrial and consumer products to prevent fire-related injury and property damage.\(^1,2\) TBBPA can be transferred to the environment during its production, usage and disposal. It has been classified as an endocrine-disrupting chemical\(^3\) because its molecular structure is similar to that of thyroxine.\(^4,5\) It can cause hepatic and kidney lesions in pregnant mice and their offspring when pregnant dams are exposed to TBBPA in the diet.\(^6\) TBBPA has been listed as a hazardous substance in the convention for the protection of the marine environment of the North-East Atlantic.

TBBPA is usually determined by chromatographic methods, such as HPLC-MS/MS (limit of detection (LOD) reported as 0.5 ng g$^{-1}$),\(^7\) UPLC-MS/MS (LOD of 0.06 ng g$^{-1}$)\(^8\) and GC-MS (LOD of 0.09 ng g$^{-1}$).\(^9\) However, these methods require complicated clean-up procedures of the samples prior to analyses and specialized instrumentation, which is tedious and time-consuming. Immunochemical methods, especially enzyme-linked immunosorbent assay (ELISA), provide a convenient and economical alternative for environmental monitoring. ELISA has been widely accepted as a screening tool for routine analysis in recent years. In an earlier study, various haptenes of TBBPA were synthesized and the polyclonal antibody (PAb) based ELISA was developed, with a half-maximum signal inhibition concentration (IC$_{50}$) of 0.87 ng mL$^{-1}$.\(^10\) The biotin-streptavidin-amplified PAb-based and the monoclonal antibody (MAB) based immunoassays for TBBPA showed an IC$_{50}$ of 0.58 ng mL$^{-1}$ and 3.87 ng mL$^{-1}$, respectively.\(^11,12\)

ELISA is a heterogeneous method in which free and antibody-bound analytes must be separated. In addition, it requires extensive pipetting, washing and incubation steps, therefore is time-consuming. A very promising alternative fluorescence polarization immunoassay (FPIA) is a shift from heterogeneous methods (with separation) to homogeneous assays (without separation).\(^13,14\) It is the most extensively used homogeneous technique, which meets the requirements of a simple, reliable, fast and cost-effective method.\(^15,16\) Briefly, FPIA is a competitive immunoassay method based on the change in the polarization of fluorescence (FP). When the small fluorescent-labeled hapten (tracer) is bound with a specific antibody, the value of FP will increase. If the sample contains unlabeled analytes, the tracer will compete for binding with the antibody and the polarization signal will fall. FPIA is a direct and ideal assay without any sample pre-treatment or washing steps.\(^18,19\)

In addition to traditional antibodies, camelid species, e.g., camels and llamas, produce a unique class of IgG known as
heavy chain IgG (HcIgG), which is devoid of light chains and the first heavy chain constant (CH1) domain. It is fully able to bind antigens despite the structure deficiency. The antigen-binding fragment, a variable domain of heavy chain domain antibody (VHH), is about 15 kDa, which retains antigen binding ability and exhibits high chemical, proteolytic and thermal stability, making it an ideal biorecognition reagent platform for small molecule analysis in general and in environmental analysis in particular.

We have developed a VHH-based ELISA for monitoring environmental and human exposure to TBBPA. In this study, the VHH against TBBPA was combined with different fluorescein-labeled tracers to develop a VHH-based FPIA for TBBPA in water samples. It provided a sensitive, specific and convenient method for the analysis and detection of TBBPA.

2. Experimental

2.1. Reagents and materials

The synthesis of six haptens (T1–T6) (Table 1) of TBBPA and production of VHH against TBBPA were described previously. The TBBPA standard was purchased from TCI Co. Ltd. (Tokyo, Japan). TBBPA derivatives and other BFR analogues were purchased from AccuStandard (New Haven, CT). Bovine serum albumin (BSA), 3,3',5,5'-tetramethylbenzidine (TMB), ethylenediamine fluorescein thiocarbamoyl (EDF), N-hydroxysuccinimide

<table>
<thead>
<tr>
<th>Structure of haptens</th>
<th>Tracer</th>
<th>(\delta_{mp})</th>
<th>IC(_{50}) (ng mL(^{-1}))</th>
<th>(\delta_{mp}/IC_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1–EDF ((R_f = 0.27))</td>
<td>127</td>
<td>102</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>T2–EDF ((R_f = 0.99))</td>
<td>87</td>
<td>163</td>
<td>0.53</td>
<td></td>
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<tr>
<td>T3–EDF ((R_f = 0.75))</td>
<td>152</td>
<td>128</td>
<td>1.18</td>
<td></td>
</tr>
<tr>
<td>T4–EDF ((R_f = 0.72))</td>
<td>14</td>
<td>ND(^a)</td>
<td>ND(^a)</td>
<td></td>
</tr>
<tr>
<td>T5–EDF ((R_f = 0.65))</td>
<td>100</td>
<td>142</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>T6–EDF ((R_f = 0.57))</td>
<td>103</td>
<td>408</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Not detectable.
(NHS) and N,N'-dicyclohexylcarbodiimide (DCC) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). N,N-dimethylformamide (DMF) was obtained from Amresco Co. (Solon, OH, USA). Tracer purity was evaluated with Sanpont thin layer chromatography (TLC) silica gel plates from Anhui Liangchen Silicon Material Co., Ltd (Anhui, China).

FPIA experiments were carried out in 0.05 M borate buffer (pH 8.0) with 0.1% sodium azide. A stock standard solution of TBBPA (1.0 mg mL⁻¹) with 0.1% sodium azide. A stock standard solution of TBBPA (50 μg mL⁻¹) was prepared in methanol and stored at 4 °C. Aqueous standard solutions of the analytes were prepared by diluting the stock standard solutions with methanol.

A VHH-based ELISA was carried out according to the procedures reported previously. Briefly, a 100 μL solution of T3-BSA (2 μg mL⁻¹) was coated on a 96-well microtiter plate at 4 °C overnight. The plate was blocked with 3% skim milk in PBS for 1 h at ambient temperature the next day. A series dilution of TBBPA (50 μL per well, 10% methanol in PBS) was added, followed by the addition of 50 μL of VHH (0.02 μg mL⁻¹) in PBST (0.05% Tween-20 in PBS). After incubation at room temperature for 1 h, the plate was washed 5 times with PBST and then 100 μL of goat anti-HA tag IgG-HRP (diluted at 1:25 000 with PBST) was added. After another incubation step and washing step, 100 μL of TMB solution (400 μL of 0.6% TMB and 100 μL of 1% H₂O₂ dilution in 25 mL citrate buffer, pH 5.5) was added into the plate and the reaction was stopped 10 min later by the addition of 50 μL of 2 M H₂SO₄. The absorbance was read at 450 nm on a microtiter plate reader.

2.2. Instruments
FPIA was carried out in glass tubes and values of intensity and polarization (mP) were read with a portable instrument Sentry-200 (Ellie, USA). Solution was mixed with a vortex finder of Dragon Lab MX-S (Dragon LAB, China). ELISA was performed in 96-well polystyrene microtiter plates (Nalge Nunc International, Denmark) and absorbance values were determined with a microtiter plate reader (Wellscan MK3, Labsystems Dragon, Finland).

2.3. Synthesis of tracers
Since the haptens employed in this study possess carboxylic acid, the tracers were conveniently synthesized by the NHS ester method. Briefly, 200 μmol of NHS and 200 μmol of DCC were added into 2 mL of anhydrous DMF containing equal moles of haptens (T1–T6). The mixture was stirred overnight at ambient temperature, followed by the addition of 1 mg of EDF. After additional stirring in the dark at ambient temperature for 3 h, an aliquot of 100 μL of the mixture was purified twice on TLC plates using a mixture of chloroform and methanol (6 : 1, v/v) as the mobile phase. The purified tracers were scraped from the plate, dissolved in methanol and stored at 4 °C. The concentration of the tracer was estimated spectrophotometrically. The tracer stock solution (10 μM) in methanol was diluted in borate buffer. The typical concentration of tracer working solution was 1.0 nM. Fig. 1 shows the schematic route for the synthesis of fluorescein-labelled T1, 3-(2,6-dibromo-4-(2-(3,5-dibromo-4-hydroxyphenyl)propan-2-yl))propanoic acid.

2.4. FPIA performance
The tracers were serially diluted in the borate buffer and the optimal dilution was determined by the fluorescence intensity signals. The fluorescence intensity of the targeted tracer dilution should be equal to about 5-fold the signal of borate buffer. VHH solution with an initial concentration of 0.2 μg mL⁻¹ was double diluted from 1/10 to 1/2560. The working tracer solution (500 μL) and VHH solution (500 μL) were mixed and fluorescence intensity signals were measured. The dilution of VHH which corresponded to approximately 70% of the maximal signal was chosen as the optimum.

For the FPIA calibration curves, 500 μL of tracer solution was mixed with 50 μL of 10-fold serially diluted TBBPA standard in a range of 0.1–10 000 ng mL⁻¹, followed by addition of 500 μL of antibody solution. After incubation at ambient temperature for 10 min, the values were read with a Sentry-200 fluorospectrometer (excitation: 485 nm, emission: 535 nm). Curves were
generated with SigmaPlot 10.0 and the IC_{50} was calculated by a four-parameter logistic equation. A series of calibration curves was constructed using different combinations of tracer/VHH and the binding activity was evaluated by comparing three parameters (δ_{mp}, IC_{50} and δ_{mp}/IC_{50}). Herein, δ_{mp} means the difference between the maximal and minimal fluorescence polarization signals. Lower IC_{50} values and higher values of δ_{mp} and δ_{mp}/IC_{50} indicate a more sensitive assay.

2.5. Optimization of FPIA

Effects of different variables including the organic solvent, pH and ionic strength on the FPIA performance (IC_{50} and δ_{mp}) were studied at ambient temperature. Methanol was added to borate buffer to form the final concentrations of 0, 5, 10, and 20% (v/v). The effect of pH was evaluated with different borate buffer solutions, ranging from pH 5.0 to 10. To estimate the influence of ionic strength, borate buffer containing NaCl in a range from 0 to 1.6% (w/v) was tested.

2.6. Cross-reactivity

After optimization, the specificity of the FPIA was evaluated by cross-reactivity (CR) through comparing the IC_{50} of TBBPA with those of several TBBPA derivatives and other analogues. The CR was calculated as follows:

\[
\text{CR} \% = \frac{[\text{IC}_{50} (\text{TBBPA})/\text{IC}_{50} (\text{tested compounds})]}{100}
\]

depicted in Fig. 2, the dilution of VHH which corresponded to approximately 70% maximum signal was chosen as the optimum. Herein, the 1/40 dilution titer was selected as the working concentration of VHH.

The tracer structure could greatly influence the immunooassay performance. As shown in Table 1, five tracers could bind with VHH to varying degrees and the weak binding of VHH with tracer T4–EDF indicates that the bromine atoms in the structure of hapten play an important role in the binding reaction. Although T3 was used as the immunizing hapten to generate VHH, a little higher sensitivity was observed by using the heterologous tracer T1–EDF (IC_{50}, 102 ng mL\(^{-1}\)) and T3–EDF (IC_{50}, 128 ng mL\(^{-1}\)) than the homologous tracer T5–EDF (IC_{50}, 142 ng mL\(^{-1}\)). Compared to T5–EDF, T3–EDF with a shorter bridge would weaken the recognition of VHH to the bridge, thus resulting in a relatively higher sensitivity. This phenomenon has been confirmed in other studies. T6–EDF showed the lowest sensitivity among the five tracers (IC_{50}, 408 ng mL\(^{-1}\)). Perhaps the fragment hapten T6 leads to more nonspecific binding than others. The selection of the optimal tracer was based on the three parameters of assay δ_{mp}, IC_{50} and δ_{mp}/IC_{50}. Among all the tested tracers, T1–EDF exhibited the lowest IC_{50} (102 ng mL\(^{-1}\)) and the highest value of δ_{mp}/IC_{50} (1.25), with a reasonable δ_{mp} (Table 1). Therefore, the tracer T1–EDF was finally selected for further studies.

3. Results and discussion

3.1. Evaluation of the efficiency of tracers and VHH

Tracer bands were scraped from the TLC plates and assessed for relative binding capacity with the VHH according to the previous report. The mP value of borate buffer is around zero and the intensity of blank buffer is about 2000. When the tracer was diluted by 10 000 times, at a concentration of 1.0 nM, the intensity was around 5-fold the signal of the borate buffer and it was used as the optimal dilution for the tracer, as in the previously reported method. If the tracer has effective binding with antibody, the mP values would decrease significantly with the dilution of antibody, otherwise, no remarkable change could be observed when the tracer cannot bind to the antibody. As shown in Fig. 2, all tracers except T4–EDF showed effective mP values (>150) which declined obviously with the dilution of VHH, indicating that those five tracers demonstrated sufficient binding ability with the employed VHH against TBBPA. According to the antibody dilution curves in Fig. 2, the dilution of VHH which corresponded to approximately 70% maximum signal was chosen as the optimum. Herein, the 1/40 dilution titer was selected as the working concentration of VHH.

The tracer structure could greatly influence the immunooassay performance. As shown in Table 1, five tracers could bind with VHH to varying degrees and the weak binding of VHH with tracer T4–EDF indicates that the bromine atoms in the structure of hapten play an important role in the binding reaction. Although T3 was used as the immunizing hapten to generate VHH, a little higher sensitivity was observed by using the heterologous tracer T1–EDF (IC_{50}, 102 ng mL\(^{-1}\)) and T3–EDF (IC_{50}, 128 ng mL\(^{-1}\)) than the homologous tracer T5–EDF (IC_{50}, 142 ng mL\(^{-1}\)). Compared to T5–EDF, T3–EDF with a shorter bridge would weaken the recognition of VHH to the bridge, thus resulting in a relatively higher sensitivity. This phenomenon has been confirmed in other studies. T6–EDF showed the lowest sensitivity among the five tracers (IC_{50}, 408 ng mL\(^{-1}\)). Perhaps the fragment hapten T6 leads to more nonspecific binding than others. The selection of the optimal tracer was based on the three parameters of assay δ_{mp}, IC_{50} and δ_{mp}/IC_{50}. Among all the tested tracers, T1–EDF exhibited the lowest IC_{50} (102 ng mL\(^{-1}\)) and the highest value of δ_{mp}/IC_{50} (1.25), with a reasonable δ_{mp} (Table 1). Therefore, the tracer T1–EDF was finally selected for further studies.

3.2. FPIA optimization

The VHH-based FPIA was optimized by evaluating the effect of physicochemical conditions on the values of IC_{50} and δ_{mp}. The optimal concentrations of VHH and T1–EDF were selected as above. Methanol is one of the widely used solvents to solubilize lipophilic compounds such as TBBPA in assay buffer and its
effect on the assay was thereby evaluated here. The assay sensitivities in 0–5% of methanol were slightly different, with IC\textsubscript{50} values changing in 100–104 ng mL\textsuperscript{-1} and \( \delta \text{mP} \) values changing in 128–131. Overlapping curves were observed in the assay buffer with NaCl contents in a range of 0–0.8%, showing an average \( \delta \text{mP} \) of 125 and IC\textsubscript{50} of 102 ng mL\textsuperscript{-1}. No significant shift was observed for the performance of FPIA at pH 7.0–9.0, as \( \delta \text{mP} \) varied from 121 to 124 and IC\textsubscript{50} varied from 95 to 97 ng mL\textsuperscript{-1}.

A typical calibration curve of competitive FPIA for TBBPA was constructed under the optimized condition of 0.05 M borate buffer (pH 8.0) containing 0.8% NaCl and 5% methanol (Fig. 3). This assay has a linear range (IC\textsubscript{20}–IC\textsubscript{80}) of 10–940 ng mL\textsuperscript{-1}, an IC\textsubscript{50} value of 90 ng mL\textsuperscript{-1}, and a LOD (IC\textsubscript{10}) of 5 ng mL\textsuperscript{-1} \( (n = 6) \) (Fig. 3). The sensitivity of FPIA for TBBPA was less than that of ELISA, which showed a linear range of 0.06–2.53 ng mL\textsuperscript{-1} (IC\textsubscript{20}–IC\textsubscript{80}), an IC\textsubcript{50} value of 0.40 ng mL\textsuperscript{-1} and an IC\textsubscript{10} value of 0.02 ng mL\textsuperscript{-1}.\textsuperscript{27} It might be caused by the FPIA format being devoid of any washing steps required in ELISA.

### 3.3. Cross-reactivity

A suitable binding of antibody with antigen can be easily affected by chemicals that are structurally related to analytes, causing false positive results.\textsuperscript{26,28} A set of important TBBPA analogues such as tetrabromobisphenol A-bis(2,3-dibromopropylether) (TBBPA-DBPE), 2,2',6,6'-tetrabromobisphenol A diallyl ether (TBBPA-BAE), hexabromocyclododecane (HBCD), 1,2-bis[pentabromodiphenyl]ethane (DBDPE), polybrominated diphenyl ethers (PBDEs) and bisphenol A (BPA) were therefore used to evaluate the cross-reactivity. The FPIA exhibited little cross-reactivity with some tested compounds including BDE-99 (3.8%), BDE-47 (4.2%), TBBPA-DBPE (6.0%), TBBPA-BAE (7.1%) and BPA (8.2%) (Table 2). The cross-reactivity of FPIA was higher than that of ELISA,\textsuperscript{27} which may result from the nonspecific binding in FPIA format without any washing and separation steps.

### 3.4. FPIA for TBBPA in water samples

To evaluate the applicability of the proposed FPIA method for screening TBBPA in real water samples, tap water and river water samples were spiked with TBBPA (20, 50 and 100 ng mL\textsuperscript{-1}) to estimate the method accuracy and precision. The average recovery and coefficient of variation (CV) values of FPIA were in the range of 73–102% and 7–14%, respectively. The spiked water was also analyzed by an ELISA based on the same nanobody which showed average recovery and CV in the range of 84–100% and 5–11%, respectively. Both the FPIA and ELISA results are in close agreement with each other (Table 3). The satisfactory accuracy and precision of this method suggested that the homogenous format FPIA based on VHH proved to be sensitive and reliable for the analysis of TBBPA in water samples.

### 4. Conclusions

This study presented a novel, rapid, sensitive, and specific alternative FPIA for TBBPA. Six haptons T1–T6 were conjugated with EDF to form different tracers. Compared to other tracers, T1–EDF (R\textsubscript{0.27}) combined with VHH showed higher binding efficiency and sensitivity for the analysis of TBBPA. The assay

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**Table 2** Cross-reactivity of FPIA for TBBPA structural analogues

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC\textsubscript{50} (ng mL\textsuperscript{-1})</th>
<th>CR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBBPA</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>TBBPA-DBPE</td>
<td>1490</td>
<td>6.0</td>
</tr>
<tr>
<td>TBBPA-BAE</td>
<td>1270</td>
<td>7.1</td>
</tr>
<tr>
<td>HBCD</td>
<td>&gt;10 000</td>
<td>&lt;1</td>
</tr>
<tr>
<td>DBDPE</td>
<td>&gt;10 000</td>
<td>&lt;1</td>
</tr>
<tr>
<td>BPA</td>
<td>1100</td>
<td>8.2</td>
</tr>
<tr>
<td>BDE-47</td>
<td>2160</td>
<td>4.2</td>
</tr>
<tr>
<td>BDE-99</td>
<td>2400</td>
<td>3.8</td>
</tr>
<tr>
<td>BDE-100</td>
<td>&gt;10 000</td>
<td>&lt;1</td>
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<tr>
<td>BDE-153</td>
<td>&gt;10 000</td>
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<tr>
<td>BDE-154</td>
<td>&gt;10 000</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5-OH-BDE-47</td>
<td>&gt;10 000</td>
<td>&lt;1</td>
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</table>

**Table 3** Recovery of TBBPA from spiked environmental water samples by both FIPA and ELISA methods \( (n = 3) \)

<table>
<thead>
<tr>
<th>Sample</th>
<th>TBBPA spiked (ng mL\textsuperscript{-1})</th>
<th>FPIA Recovery (%)</th>
<th>FPIA CV (%)</th>
<th>ELISA Recovery (%)</th>
<th>ELISA CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xiaoqing river water</td>
<td>0</td>
<td>ND\textsuperscript{a}</td>
<td>ND\textsuperscript{a}</td>
<td>ND\textsuperscript{a}</td>
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<tr>
<td>20</td>
<td>73</td>
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<td>84</td>
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<td>100</td>
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<tr>
<td>500</td>
<td>91</td>
<td>14</td>
<td>94</td>
<td>11</td>
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<tr>
<td>Tap water</td>
<td>0</td>
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<td>ND\textsuperscript{a}</td>
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<tr>
<td>500</td>
<td>102</td>
<td>12</td>
<td>100</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

\( \text{ND} \) Not detectable.

**Fig. 3** The standard curve of FPIA for TBBPA under the optimized condition. mP and m\textsubscript{P\textsubscript{0}} separately indicate the fluorescence polarization in the presence and absence of TBBPA in assay buffer. Error bars indicate standard deviations from six replicate measurements.

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IC\textsubscript{50} was 90 ng mL\textsuperscript{-1} and cross-reactivity was indistinctive. The recovery and coefficient of variation for analyzing TBBPA in water samples were as good as an ELISA method. Even though a lower sensitivity and a less selectivity of the FPIA for TBBPA were obtained than those of an ELISA, the former method showed significant advantages in assay time, within 20–30 min for an analysis, by avoiding several washing steps in an ELISA method, typically more than 2 h for an analysis. The VHH-based FPIA has high potential for wide applications in environmental analysis.

Acknowledgements

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