Development of an indirect competitive ELISA for the detection of acenaphthene and pyrene

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ABSTRACT
Polycyclic aromatic hydrocarbons (PAHs) have mutagenic and carcinogenic properties. Acenaphthene and pyrene were members of 16 PAHs which were listed as the priority pollutants in water environment by US Environmental Protection Agency. The reported instrumental methods and immunoassays did not meet the need for simple and sensitive detection of acenaphthene and pyrene. In this study, a monoclonal antibody having high affinities with acenaphthene and pyrene was produced and an indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed for sensitive detection of acenaphthene and pyrene in water sample. The linear range of the assay was between 3.53 and 41.98 ng mL\textsuperscript{-1}. The sensitivity was 12.17 ng mL\textsuperscript{-1} which was more sensitive than those of reported ELISAs. The average recovery of acenaphthene and pyrene from three kinds of water samples was 99.08\% and 98.45, respectively. The developed ELISA could be used for sensitive detection of acenaphthene and pyrene in water samples.

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1. Introduction
Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds and composed of multiple fused aromatic rings (Dost & İdeli, 2012). They are considered as widespread contaminants in air, soil and water (Masih, Masih, & Taneja, 2012). PAHs have properties of mutagenic and carcinogenic, resulted in lung, skin and prostate cancer of human and harmed people’s endocrine system (Isobe et al., 2007; Kim, Jahan, Kabir, & Brown, 2013; Wenzl, Simon, Anklam, & Kleiner, 2006). Acenaphthene and pyrene were listed as the priority PAHs in water environment by US Environmental...
Protection Agency (EPA) (Zhang & Tao, 2009). Therefore, it is necessary to develop a sensitive method for the detection of acenaphthene and pyrene in environment.

A great number of instrumental methods have been developed for the detection of PAHs, such as high-performance liquid chromatography (HPLC) (Dost & İdeli, 2012), HPLC with fluorescence (Spier, Bromage, Harris, Unger, & Kaattari, 2009), HPLC with fluorescence and diode-array detectors (Huang, Wei, Song, Chen, & Luo, 2013), gas chromatography/mass spectrometry (GC/MS) (Aragón, Toledano, Vázquez, Villén, & Cortés, 2015; Barco-Bonilla, Vidal, Frenich, & Romero-González, 2009) and atmospheric pressure gas chromatography (Domeno, Canellas, Alfaro, Rodriguez-Lafuente, & Nerin, 2012). However, these techniques require elaborate sample preparation and skilled analysts. Few immunoassays have also been reported, such as fluorescence polarization immunoassay for benzo[a]pyrene, naphthalene and anthracene (Spier et al., 2009), competitive ELISA for pyrene, chrysene, benzo[a]pyrene, benzo[a]anthracene and indeno [1,2,3-cd]pyrene (Meng et al., 2015), intramolecular energy transfer probe (Kupstat, Knopp, Niessner, & Kumke, 2010), multichannel surface plasmon resonance (SPR) biosensor (Dostálek, Přibyl, Homola, & Skládal, 2007), photoelectrochemical immunosensor (Kang et al., 2011), electrochemical immunosensor and surface infrared immunosensors for benzo[a]pyrene (Ahmad & Moore, 2012; Boujday, Nasri, Salmain, & Pradier, 2010).

Monoclonal antibody (mAb) based enzyme-linked immunosorbent assay (ELISA) has been widely applied in pollutant detection in environment (Li et al., 2014; Meng et al., 2015; Spier et al., 2009). Compared with instrumental methods, ELISA does not require sophisticated instrumentation, skilled analysts and labor intensive sample preparation. Several ELISAs based on mAb specific for benzo[a]pyrene and dibenzothiophene were developed (Karsunke et al., 2011; Matschulat, Deng, Niessner, & Knopp, 2005; Spier et al., 2009). However, the affinities of these ELISAs for acenaphthene and pyrene were not higher (Table 1). In this study, we produced a mAb and developed an ELISA based on the mAb for sensitive detection of acenaphthene and pyrene in water samples.

2. Materials and methods

2.1. Materials

5-Acenaphthylenecarboxylic acid (ALA), Freund’s complete adjuvant (FCA), Freund’s incomplete adjuvant (FIA), polyethylene glycol (PEG 1450), HT Media Supplement

### Table 1. Sensitivity comparison of the proposed ic-ELISA with reported ic-ELISAs for acenaphthene detection.

| Method     | Detected target | IC₅₀ of target (ng mL⁻¹) | Cross-reactivity with acenaphthene | IC₅₀ of acenaphthene (ng mL⁻¹) | Reference                                      |
|------------|-----------------|--------------------------|-----------------------------------|-------------------------------|------------------------------------------------|
| Ic-ELISA   | benzo[a]pyrene  | 8.9                      | <1%                               | >890.00                       | Scharnweber, Fisher, Suchanek, Knopp, and Niessner (2001) |
| Ic-ELISA   | benzo[a]pyrene  | 4.84                     | 1%                                | 484.00                        | Matschulat et al. (2005)                         |
| Ic-ELISA   | benzo[a]pyrene  | 0.31–0.58                | 1.6–0.9%                         | 19.38–64.44                  | Karsunke et al. (2011)                          |
| Ic-ELISA   | benzo[a]pyrene  | 0.6835                   | <0.1%                             | >683.50                      | Meng et al. (2015)                              |
| Presented ic-ELISA | acenaphthene | 12.17            | 100%                               | 12.17                         | –                                              |
(50×) Hybri-Max™, HAT Media Supplement (50×) Hybri-Max™, bovine serum albumin (BSA), RPMI 1640, egg albumin (OVA), N-Hydroxysuccinimide (NHS) and N, N-Dicyclohexylcarbodiimide (DCC) were purchased from Sigma (USA). Penicillin–streptomycin solution and N, N-dimethyl Formamide (DMF) were obtained from Beijing Ding Guo Changsheng Biotechnology Co., Ltd (China). Anthracene, acenaphthylene, acenaphthene, benzo[a]pyrene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[g,h,i]perylen, chrysene, fluoranthene, benzo[k]fluoranthene, dibenz[a,h]anthracene, fluorene, indeno[1,2,3-cd]pyrene, phenanthrene, pyrene and naphthalene were purchased from J&K Scientific (Beijing, China). Horseradish peroxidase conjugated goat anti-mice IgG (HRP-IgG) was supplied by Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd (Beijing, China). Microtiter plates and cell culture bottles were purchased from Costar Group, Inc. (USA). Special Placenta Bovine Serum (FBS) was obtained from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd (Hangzhou, China). Other reagents were analytical grade and purchased from Sinopharm Chemical Reagent Beijing Co., Ltd (Beijing, China).

2.2. Cell line and animal

Mouse myeloma cells (SP2/0) were purchased from the Chinese Academy of Sciences (Shanghai, China). BALB/c mice were obtained from Changchun Institute of Biological Products Co., Ltd (Changchun, China).

2.3. Buffers and solutions

Phosphate buffer saline (PBS) solution: 10 mmol L\(^{-1}\) sodium phosphate buffer (pH 7.4) containing 140 mmol L\(^{-1}\) NaCl. Washing buffer (PBST): PBS solution containing 0.05% (v/v) Tween 20. Coating buffer (carbonate buffer solution, pH 9.6): 0.05 mol L\(^{-1}\) carbonate buffer. TMB solution: 0.01% TMB (W/V) and 0.005% (W/V) urea hydrogen peroxide in a citrate buffer (pH 5.0). Stop solution: 2 mol L\(^{-1}\) sulfuric acid.

2.4. Preparation of acenaphthene-protein conjugates

The immunizing antigen (ALA–BSA) was prepared by conjugating ALA to BSA on the basis of mixed anhydride method according to literature (Chen, Zhuang, Yang, & Ji, 2013; Feng et al., 2013; Yuan et al., 2014). In brief, 40 mg ALA was dissolved in 1 mL of DMF, then 53 μL of 3-butylamine was added into the solution and the mixture was stirred for 20 minute at 4°C. After 29 μL isobutylchloroformate was added into the solution, the mixture was mixed 4 hour at 4°C. Subsequently, the mixture solution was added dropwise to 2.5 mL PBS solution containing 100 mg BSA, and then the mixture was gently stirred overnight at 4°C. The final mixture was dialyzed in 0.01 mol L\(^{-1}\) PBS for three days at 4°C to remove residual free ALA and some chemical reagents. The preparation procedure of ALA–OVA was the same as the method of ALA–BSA. The reagent-treated BSA or OVA was executed by the same method only without ALA. The conjugations of ALA–BSA and ALA–OVA were confirmed using ultraviolet-visible spectroscopy.
**2.5. Hybridoma selection**

All animal experiments were conducted in accordance with the guide for the care and use of laboratory animals (Gaber et al., 2010). Eight-week-old female BALB/c mice were immunized with ALA–BSA via hind footpad (Meng et al., 2015). Briefly, 100 µg of immunizing antigen (in 25 µL PBS) with 25 µL of FCA was emulsified and injected hind footpad for each mouse in the first immunization. The mice were boosted with same volume of FIA and half dosage immunizing antigen in the same manner every 1 week. Four days after the third immunization, blood samples were taken from the tail vein and the sera was determined to identify the presence of antibody against ALA by indirect ELISA using ALA–OVA as coating antigen. The final booster was administered via peritoneal cavity injection with 100 µg immunizing antigen in PBS without adjuvant. Three days after the last immunization, cell fusion was performed in accordance with literature (Wang et al., 2009). In brief, the splenocytes were mixed with SP2/0 myeloma cells in a ratio of 5:1 in the presence of PEG. The fused cells were cultured in RPMI 1640 medium containing 2% HAT, 20% (v/v) FBS and 1% penicillin–streptomycin at 37°C in an atmosphere of 5% CO₂. The medium was half exchanged every four days. After 12 days, the cell culture supernatants was detected by indirect competitive ELISA, the positive hybridoma which showed the characteristics of binding to acenaphthene but not with the BSA themselves were cloned four times with limited dilution method.

**2.6. Production and purification of mAb**

The mature female BALB/c mice were injected liquid olefin via peritoneal cavity for harvest ascites. One week later, the mice were injected about 10^7 hybridoma cells per mouse via peritoneal cavity. After 10 days later, the ascites was collected through the needle of 20 mL injector and purified with HiTrap Protein G column by AKTA Purifier 100 equipment. The subtype identification of the mAb was performed according to the instruction of the mouse mAb isotyping reagents kit (sigma). The purified mAb was identified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Zhang et al., 2014) and then kept at −80°C.

**2.7. Procedures of the ic-ELISA**

The coating antigen ALA–OVA (100 µL/Well, 1 µg mL⁻¹) was added into 96-well microtiter plates and incubated 2 hour at 37°C. The microtiter plates were washed three times with PBST (200 µL/Well) and then blocked with 1% BSA for 1 hour at 37°C. After washing three times with PBST, 50 µL sample solution (or 50 µL acenaphthene standard solution) and 50 µL diluted mAb (1: 32,000 in PBS) was added in well and incubated 1 hour at 37°C. After another washing step, 100 µL goat anti-mouse IgG-HRP (1:5000 in PBS) was added and incubated 1 hour at 37°C. The microtiter plates were washed again, and 100 µL TMB solution was added to each well and reacted for 10 minute at 37°C. The enzyme reaction was terminated with 50 µL of stop solution. Finally, the absorbance was measured at 450 nm with Epoch Microplate Spectrophotometer.

Calibration curve was drawn using the relationship between the values of inhibition and the concentration of acenaphthene. The inhibition value was calculated using the
following formula (Feng et al., 2013; Meng et al., 2015): Inhibition (%) = \[1 - \frac{B}{B_0}\] × 100 (B was the corresponding values at different concentration of acenaphthene, B_0 was the OD_{450} value without acenaphthene).

2.8. Cross-reactivity (CR)
EPA-designated 16 PAHs were used for CR of the assay. The CR values were measured through the following formula (Yuan et al., 2014): CR% = (IC_{50} of acenaphthene/IC_{50} of interferent) × 100.

2.9. Analysis of spiked samples
Three water samples including commercial mineral water (local supermarket), Nanhu lake water (Changchun, Jilin province, China), and Yitong river water (Changchun, Jilin province, China) were collected and filtered through 0.45 μm Millipore filter. Each sample was first analyzed using the assay and then spiked with acenaphthene and pyrene at concentrations of 6.25, 12.5 and 25 ng mL^{-1}, respectively. The recoveries were calculated according to literature (Meng et al., 2015).

3. Results and discussion
3.1. Analysis of the immunizing antigen and coating antigen
Ultraviolet-visible spectroscopy was usually used to identify the synthesis of antigen (Chen et al., 2013; Meng et al., 2015; Yi, Wang, Li, Zhu, & Ying, 2012). The ultraviolet-visible spectroscopy results were shown in Figure 1. The maximum absorption peaks of BSA, reagent-treated BSA, immunizing antigen (ALA–BSA) and ALA were 228, 238, 247 and 295 nm, respectively (Figure 1(a)). The maximum absorption peaks of OVA, reagent-treated OVA, coating antigen (ALA–OVA) and ALA were 228, 232, 236 and 295 nm, respectively (Figure 1(b)). These results proofed that ALA was successfully conjugated with BSA and OVA.

3.2. Characteristics of the mAb
Following the fusion of splenocytes and SP2/0, one positive hybridoma was selected using limiting dilution method. The mAb was collected from ascites. As shown in Figure 2, there are two obvious bands in the purified mAb, which are the heavy chain and light chain. The molecular weight of heavy chain and light chain were approximately 50 and 25 kDa (Figure 2, lane 1), respectively. The subtype of the mAb was IgG2b identified using mouse mAb isotyping ELISA reagents kit (Figure 3).

3.3. Sensitivity and calibration curve
The inhibition cure of acenaphthene was shown in Figure 4. The linear range of the assay was from 3.53 to 41.98 ng mL^{-1} (IC_{20}–IC_{80}). IC_{50} value is the concentration of small molecular which generates a 50% inhibition of antibody binding to the antigen.
It was considered a sensitivity measure of the mAb. As shown in Figure 4, the IC<sub>50</sub> value was calculated to be 12.17 ng mL<sup>-1</sup>, which was lower than those of reported ELISAs (Karsunke et al., 2011; Matschulat et al., 2005; Meng et al., 2015; Scharnweber et al., 2001) (Table 1).

### 3.4. Cross-reactivity studies

The specificity of the mAb was determined using 16 EPA-designated PAHs and the result was shown in Table 2. The mAb showed high affinity with acenaphthene (100%) and pyrene (108.66%), low affinity with fluorene (4.25%), phenanthrene (8.00%), anthracene (24.51%), fluoranthene (5.93%) and benzo[a]pyrene (30.02%). The CR with acenaphthylene, chrysene, benzo[a]anthracene, benzo[b]fluoranthene,
benzo[k]fluoranthene, naphthalene, indeno[1,2,3-cd]pyrene, benzo[g,h,i]perylene and dibenz[a,h]anthracene was less than 1%.

3.5. Analysis of spiked samples

In order to evaluate the applicability of the developed ic-ELISA, water samples of lake water, river water and mineral water were spiked with acenaphthene and pyrene at 0, 6, 12.5 and 25 ng mL$^{-1}$, respectively. As shown in Table 3, the average recoveries of acenaphthene, pyrene and the mixture of acenaphthene and pyrene were 99.08%, 98.45% and 100.44%, respectively. All coefficients of variation (CVs) are within 7.98%, indicating a good reproducibility. The results indicated that the water matrix did not influence the assay.
Figure 4. Inhibition curve of the developed ELISA. The curve was obtained by the relationship between the inhibition values and the concentration of acenaphthene. Data represent means ± standard deviations from three separate experiments.

Table 2. Specificity of mAb with 16 EPA-designated PAHs.

| Compound             | Structure | IC$_{50}$ (ng mL$^{-1}$) | Cross-reactivity (%) |
|----------------------|-----------|--------------------------|----------------------|
| Acenaphthene         |           | 12.17                    | 100.00               |
| Pyrene               |           | 11.20                    | 108.66               |
| Benzo[a]pyrene       |           | 40.54                    | 30.02                |
| Anthracene           |           | 49.65                    | 24.51                |
| Phenanthrene         |           | 152.13                   | 8.00                 |
| Fluoranthene         |           | 205.40                   | 5.93                 |
| Fluorene             |           | 286.61                   | 4.25                 |
| Naphthalene          |           | >1217.00                 | <1.00                |
| Acenaphthylene       |           | >1217.00                 | <1.00                |
| Benzo[a]anthracene   |           | >1217.00                 | <1.00                |
| Chrysene             |           | >1217.00                 | <1.00                |
| Benzo[b]fluoranthene |           | >1217.00                 | <1.00                |
| Benzo[k]fluoranthene |           | >1217.00                 | <1.00                |
| Indeno[1,2,3-cd]pyrene|           | >1217.00                 | <1.00                |
| Dibenz[a,h]anthracene|           | >1217.00                 | <1.00                |
| Benzo[g,h,i]perylene |           | >1217.00                 | <1.00                |
4. Conclusion

Based on the produced mAb, an ic-ELISA was developed for the detection of acenaphthene and pyrene. The sensitivity of the assay is higher than those of the reported ELISAs. The recoveries showed that the matrix of water samples did not effect on the assay. The developed ic-ELISA can provide a valuable method for sensitive detection of acenaphthene and pyrene in water samples.

Disclosure statement

No potential conflict of interest was reported by the authors.

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