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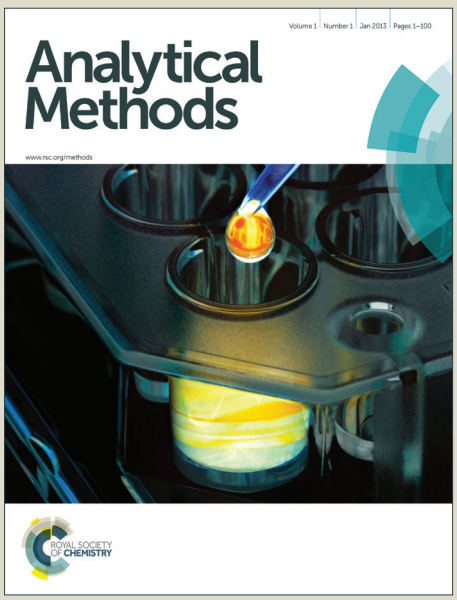

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Fluorescence polarization immunoassay method for bisphenol A residue in environmental water samples based on monoclonal antibody and 4'-(Aminomethyl) fluorescein

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Keywords: Bisphenol A (BPA), Fluorescence polarization immunoassay (FPIA), 4'-(Aminomethyl) fluorescein (AMF), Monoclonal antibody.

Abstract

Based on a sensitive monoclonal antibody against bisphenol A (BPA) and a new tracer named BVA-AMF, a homogeneous fluorescence polarization immunoassay (FPIA) was developed and applied in the determination of bisphenol A in environmental water samples. BVA was selected as the hapten to couple with bovine serum albumin and the conjugate was used as the immunogen for the monoclonal antibody production. Three fluorescein-labeled BVA tracers with different structures (BVA-AMF, BVA-EDF, BVA-lysFITC) were synthesized. Under the same optimal conditions, BVA-AMF showed most sensitivity for FPIA and the detection of BPA was with a limited detection of 5.60 ng/mL, IC_{50} of 140 ng/mL and a dynamic range of 11.32-904.21 ng/mL approximately. In this assay, several similar compounds were shown of little significantly with the cross reactivity being less than 0.15%. Four different kinds of water samples were analyzed, with recoveries being 87.91%-114.28%. The detection standard curve for BPA exhibited good linearity (R^2=0.9913, n=3). Compared with ELISA and HPLC methods, FPIA showed reliability and high correlation with ELISA of 0.9964 and HPLC of 0.9971. The immunoassay technique demonstrated that was responsible for detection of BPA in authentic environmental water samples.

1. Introduction

In recent years, numerous researches indicated that Bisphenol A (BPA) have high potential as endocrine disruptors in humans and wildlife. It was considered that it has an estrogenic reactivity and effects on the generation, immunity and nerve systems[1]. Bisphenol A is a crucial organic chemical material, primarily used as plasticizer, fire retardant, coating in the production of polycarbonate plastic and epoxy resins[2], and plastic in food packaging or other packagings used as lining surface coatings[3]. Upon exposure to heat, acid or base, ester bonds that link the BPA monomers in these plastic or resin materials will be hydrolyzed, thus BPA was released from the materials containing BPA additive in water or soil[4]. The negative health effect would be brought into people or animals when they contacting the waste water or soil. Thus, an

increasing amount of attention has been called as the reports of BPA in the environment of residual growing[5, 6]. Many countries have declared restrictions and regulations for BPA. The European Union (EU) believes that bisphenol A-containing baby bottles will induce precocious puberty. In China, the department of health announced that bisphenol A to be prohibited for its use in bottles for infant feeding. Therefore, there is a desperate need for a rapidly effective and easily operating method to deal with the problem.

Thus far, a large number of analytical methods have been available for determination of bisphenol A, including chemical methods, biological methods[7-9] and instrument determinations[3, 10, 11]. Recently, molecular imprinting of detection has become more popular. Generally, high-performance liquid chromatography (HPLC) and gas chromatography (GC) are accurate and reliable but expensive, time-consuming and improper for a large amount of sample analysis. Immunoassays have been widely studied and used for the detection of BPA, many researched reports are about antibody production and development of immunoassay method and established ic-ELISA[12], ELISA[13, 14], CLEIA[15], FLISA[16], FPIA[17] methods which were used for determination based on polyclonal antibody, monoclonal antibody[18] or single antibody. ELISA[19] and CLEIA[1] are heterogeneous methods which need 2-3h and several washing times, while FPIA is a homogeneous method which needs no separation or washing steps but just a short time of 10min. FPIA is a practical tool for rapid analysis of food and environmental samples. It is mainly based on a reliable fluorescein and a sensitive antibody[20, 21]. Wu etc. had set up a FPIA method based on fluorescein (BVA-EDF) and polyclonal antibody, and the paper was published on Immunological Investigation[22]. Therefore, there is a big demand to develop another sensitive FPIA for determination of BPA within a new fluorescein and sensitive monoclonal antibody.

In this work, we have successfully synthesized a new tracer named BVA-AMF using hapten and AMF combining[23], and generated the sensitive monoclonal antibody against BPA. The fluorescence polarization immunoassay (FPIA) was established based on fluorescein (AMF) and monoclonal antibody. However, a sensitive

monoclonal antibody and AMF has not been used for bisphenol A detection. The aim of the present work was to develop a rapid, costless and sensitive immunoassay for determination of BPA in the water from the natural environment. Moreover, under the optimal conditions of tracers, tracer dilution and antibody dilution, a homogeneous competitive fluorescence polarization immunoassay was applied to determination of authentic samples, and also validated by two reference methods (ELISA and HPLC). The interpretation of result indicating FPIA for determination of BPA was rapid, sensitive and credible.

2. Materials and methods

2.1 Reagents

Bisphenol A (BPA), 4,4-Bis(4-hydroxyphenyl)-valeric acid (BVA), N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), triethylamine and 1Ethyl3(3dimethyllaminopropyl)carbodie hydrochide (EDC) were obtained from Aladdin. Fluorescence isothiocyanate (FITC), 4'-(Aminomethyl) fluorescein (AMF) were obtained from Invitrogen. Freund's complete adjuvant, incomplete adjuvant and bovine serum albumin (BSA) were purchased from Sigma. N, N-dimethylformamide (DMF) was purchased from Damao Reagent Company in Tianjin and methanol was purchased from Merck KGaA in Darmstadt of Germany. In this work, all chemicals were of analytical reagent grade, and ultrapure water was used throughout all the experiments.

Buffer solutions: Borate buffer (BB, pH = 8.0): 0.48 g of $Na_2B_4O_7.10H_2O$, 0.05 g of NaCl in 500 mL H_2O.

2.2 Instruments

Fluorescence polarization immunoassay procedure was performed on a FPIA Sentry 200 by elli (Diachemix). Ultraviolet spectra was recorded on a UV-Vis Spectrophotometer (UV2450, Shimadzu, Japan). HPLC analyses were performed by use of a LC-20A (Shimadzu, Japan).

2.3 Preparation of immunogen

30.5 mg of NHS and 52.2 mg of EDC were respectively dissolved in 2 mL of

H_2O, and 62.0 mg of BVA was dissolved in 2 mL of DMF. The above three solutions were mixed for 24h with stirring at room temperature, and then added with 5 mL PBS dissolved with 127 mg BSA. The new mixture was stirred for 24h at room temperature in the dark. The reaction solution was dialyzed against PBS for three days after centrifugation, freezed for drying and stored at -20 ℃ until use. BCA Protein Quantitation Kit was used to measure protein concentration of BVA-BSA and the complete immunogen was authenticated by ultraviolet spectra.

2.4 Production and characteristics of MAb against BPA

BVA-BSA conjugate was used as the immunogen through the immunization procedure. Five 8-week-old BALB/c female mice were immunized by intraperitoneally injected with 100 μg BVA-BSA, emulsified with an equal volume of Freund's complete adjuvant. Booster injections were given with an equal volume of incomplete adjuvant emulsion in the same manner every two weeks. The serum was collected at the seventh day after the third immunization. An indirect competitive enzyme-linked immunosorbent assay was used to check the serum titer. Until 1.6×105 of the serum binding to BVA-PLL (poly-L-lysine), the mouse could be donated for hybridoma production. The spleen cells of the immunized mouse were fused with the myeloma cells SP2/0 with PEG1450 at a ratio of 5:1 after the mouse being injected intravenously with 50 μg BVA-BSA three days before the cell fusion. After the fusion, the fused cells were dispensed in the 96-well cell culture plates filled with complete medium (RPMI1640 with 20% FBS/HAT medium). Positive hybridomas secreting the MAb against BPA were screened by an indirect competitive ELISA and cloned four times by limiting dilution. Stable and valuable antibody-producing clones were largely generated with the ascites. Finally, the monoclonal antibody against BPA was gained and purified by ammonium sulfate precipitation and stored at -20 ℃ until use. The subtype of MAb against BPA was identified by using IsoQuick Kit for Mouse Monoclonal Isotyping.

2.5 Synthesis of fluorescein-labeled tracers

In this syudy, three fluorescent tracers (BVA-AMF, BVA-lysFITC, BVA-EDF) were designed for the detection of BPA. The fluorescein thiocarbamyl

ethylenediamine (EDF) was synthesized from FITC and ethylenediamine dihydrochloride as previously described. The lysFITC with active amino-group was synthesized from FITC and Lysine in the same way[24]. The synthesis tracers were made by pre-activation of carboxyl-group of BVA and its conjugation with amino-group of Fluorescein derivatives (AMF, EDF, lysFITC). In brief, 10.9 mg (38 μmol) of BVA was mixed with 10.4 mg (90 μmol) N-hydroxy succinimide (NHS) and 18 mg (87 μmol) dicylohexylcarbodiimide (DCC) in 1 mL dimethylformamide (DMF) and was incubated overnight at room temperature under stirring. 50 μL (2 μmol) of the resulting solution of activated BVA and 10 μL triethylamine then were added to 1 mg (2.5 μmol) AMF. The reaction mixture was mixed and a yellow-orange solution was formed. After 3 h the reaction mixture of 10 μL was purified by thin-layer chromatography (2.5x7.5 cm; Kieselgel 60, Merck), by using $CHCl_3$:CH_3OH:CH_3COOH (4:1:0.1, v/v) as the mobile phase. The main yellow band for tracer BVA-AMF with Rf = 0.8 and Rf = 0.9 was extracted in 100 μL methanol and stored at −20 ºC. The synthesis of tracers BVA-EDF and BVA-lysFITC were made in the same way by use of 50 μL activated BVA and 1 mg amino-derivative of Fluorescein. The main yellow bands for BVA-EDF at Rf = 0.6 and for BVA-lysFITC at Rf = 0.2 were collected and used as tracers.

With these tracers, TLC was used for identifying and purifying chloroform to be used: methanol: acetic acid glacial (4:1:0.1, v/v) as eluent. The main yellow bands were collected on the silica gel plate (BVA-AMF: Rf = 0.8, 0.9; BVA-lyFITC: Rf = 0.2; BVA-EDF: Rf = 0.6). If needed, a second TLC would be performed for separation of the tracers. With the good tracer and a more sensitive FPIA developed, Rf = 0.99 of BVA-AMF was purified in the second TLC process.

2.6 FPIA procedure

Fluorescence polarization immunoassay experiments were based on Borate buffer (0.05 M, pH = 8.5), and standard working solution or cross-reaction working solution was 10% methanol in ultrapure water. For more sensitive fluorescence polarization method, optimization of tracers and antibody concentration must be the essential steps prior to FPIA performance. Different dilution of antibody solution and

different concentration of standard working solution (0, 1, 3, 10, 30, 100, 300, 1000, 3000, 10000 ng/mL) were prepared for FPIA experiment. Twenty microliter of the standard or sample solution, 500 μL of the trace working solution and 500 μL of the antibody working solution were mixed in the glass culture tube. The fluorescence polarization was measured after 10min of incubation and stirring at room temperature. Twenty microliter of BB was used in place of the standard as the blank control. The competitive assay was also used to check the chemicals whose structures were similar to BPA (BVA, BPS, Phenolphthalein, Phenol, Hydroquinone, Benzene) and evaluated as the cross-reaction rate (CR). Cross-reactivity was calculated according to the following equation: CR% = [IC_{50} (BPA)/ IC_{50} (structurally related compounds)] × 100%.

2.7 Analysis of spiked samples

Four types of environmental water were analyzed in this study. The water from the Pearl River tributary, the water from the central lake in Guangzhou Higher Education Mega Center, the water from the lake in Guangdong University of Technology and the water from the laboratory tap of the authors were collected, 10 mL of every water samples were centrifuged for 20min at 6000rpm, 1M HCl or 1M NaOH was chosed to adjust pH value to neutral, filtered by 0.22μm microporous filtering film, stored in 4℃ until test. To evaluate the recovery of the FPIA established, four types of environmental water were spiked BPA at levels of 0, 10, 50, 100 ng/mL and determined by FPIA, respectively, water samples without BPA was used as a negative controls.

2.8 Evaluation of authentic samples by FPIA, ELISA and HPLC

ELISA and HPLC were selected to determine the standard and samples were used to validate the FPIA to be developed. Correlation studies between methods were also performed on the same spiked solutions. ELISA was carried out as previously done by our co-workers. The conditions were as following: 0.25 μg/mL of coating antigen, 1:8000 dilution of MAb, 1:5000 dilution of goat anti-mouse IgG-HRP, and the results were recorded at 450 nm and 630 nm with an RT-200C microplate reader. HPLC was performed on a LC-20A equipment with an EclipseXDB2-C18 column by

using a mixture of methanol and water (60:40, v/v) as the mobile phase at a flow rate of 1.0 mL/min at 30 ℃. The detection wavelength was 221 nm, and the injection volume was 50 μL. The retention time was about 9.53min.

3. Results and discussion

3.1 Synthesis of immunogen and identification of MAb

BPA is a regular molecular, which bears two phenolic hydroxyl moieties. BPA was not suitable to combine BSA and to keep its characteristic moiety. From our co-workers' previous work experience, BVA was selected as the hapten to produce the immunogen in this study. The selected hapten was effectively available while complicated organic synthesis was not required. The active carboxyl of BVA and the amino of BSA were coupled through the active ester method, and the conjugate synthesis was identified by ultraviolet spectrum. The peak of BVA-BSA was stronger than the similar peak of BSA at 278 nm with the same protein concentration (Fig.1). BVA was considered to own maximum absorption peak wavelength. Therefore from the Figure1 and it explanation, it was concluded that the immunogen was a satisfactory conjugate for the immunization.

The monoclonal antibody against BPA named 5B8 was generated from ascites in sensitized BALB/c. The subtype of MAb against BPA was identified to be IgG1 and Kappa light chain by using IsoQuick Kit for Mouse Monoclonal Isotyping. ELISA was used for analyzing the titer and the LOD value of the MAb. The results indicated that the MAb was stable and sensitive for detection of BPA. Therefore, the MAb was chosen for the subsequent investigation.

3.2 Synthesis and characterization of tracers

To achieve optimum sensitivity of the homogeneous assay technique, three different types of tracers (BVA-AMF, BVA-EDF, BVA-lysFITC) were synthesized with fluorescein and hapten, which provide different fluorescent signals. Through the active ester method, the hapten was covalently bound to fluorescein. The tracers were isolated by TLC procedure and the main yellow bands were collected. All the tracers were checked for antibody binding. After separation by TLC, the main yellow band of

BVA-AMF which showed sufficient binding power with monoclonal antibody was purified, and FPIA results indicated that BVA-AMF was selected as the tracer for further investigation. The results showed that BVA-AMF of Rf = 0.99 was with good affinity to the 5B8 MAb. That is to say, the main yellow band of BVA-AMF (Rf = 0.99) showed sufficient binding power with the monoclonal antibody, and this tracer was synthesized successfully and selected for the continuing studies.

FPIA was a homogeneous immunoassay, whose performance was based on antigen-antibody interactions. It was a competitive method involving detection of the reaction mixture containing the sample, fluorescent-labeled tracer and specific antibody. The experimental conditions are significant elements for the FPIA. Therefore, the optimization is important to enhance the sensitivity and reliably of the method, which was critical for an approving FPIA. In this work, the tracer selection and antibody dilution optimization were decided as assay parameters.

The FPIA instrument used in this work was Sentry 200 portable system, the test depending on two parameters (mP and intensity). The mP value of borate buffer is about 25 and the intensity of blank buffer is around 16000. So the intensity of free tracer was about 10-fold the signal of the borate buffer, which showed that the tracer had good signal and checked with binding antibody. Three types of tracers and a serious of dilutions of tracers were detected with the method of FPIA. BVA-lysFITC, BVA- EDF and BVA-AMF were respectively bound with monoclonal antibody and the selective tracers were tested for identifying the optimal condition. With the data showed in Fig.2, it could be seen that BVA-AMF was the best for the current experiment and the dilution was 1:12500. Appropriate tracer with high signal was very important for the sensitivity of the FPIA.

The dilution of antibody was an essential factor for the immunoassay method, because the sensitivity, reliability and the cost were great related to the antibody. The combination of tracer and antibody would change the value of the mP. If more antibodies and tracers were mixed, mixture molecules would be bigger because of more antibody binding, so that the mP was to be amplified. In this study, monoclonal antibody dilution of 1-10 μL/mL (MAb/BB) were checked and analyzed. The mP of

antibody and tracer (no standard or sample) about 200 was appropriate and it can guarantee the sensitivity of the FPIA. As showed in Fig.3, the monoclonal antibody dilution of 5 μL/mL (MAb/BB) was appropriate for this experiment.

Under the optimal assay condition, the competitive inhibition curve was established. The sensitivity of the FPIA was studied and represented by IC_{50} and limit of detection. As showed in Fig.4, the detection standard curve for BPA exhibited a good linearity (R^2 = 0.9913, n = 3) with the concentration of BPA from 11.32-904.21 ng/mL. The IC_{50} was calculated to be 140.60 ng/mL and LOD was 5.60 ng/mL. Then the method was applied to sample detection.

3.3 Specificity

To evaluate FPIA of determination of BPA, cross-reactivity was measured and calculated by IC_{50} of related compounds and BPA. Six similar analytes structurally related to BPA were selected for test and analysis. As shown in the form, the results of BPA and BVA were similar. It indicated that the antibody was specified to BPA. As was shown in Table 1, BVA had a high cross-reactivity, of about 106%, a lower IC_{50} value and higher affinity. It was reasonable that BVA had been used as the hapten to carry protein BSA for preparing immunogen. The other similar compounds have no significant difference in structure and function with BPA. The CR value of the similar compounds showed in the form were extremely low (<0.15%). In a word, the monoclonal antibody against BPA was specific and sensitive.

3.4 Analysis of spiked samples

The optimized fluorescence polarization immunoassay method developed above was applied to regulate BPA spiked in environmental water. Four types of waters were pretreated by being placed, centrifuged and adjusted to neutral. They were then added with different amounts of BPA. The recoveries were calculated and showed in the Table 2. In Table 2, it was indicated that the spiked recoveries ranged from 87.91%-114.28% by FPIA and from 85.40%-104.18% by HPLC, while recoveries of ELISA were 92.60%-112.70%. The coefficient of variation was less than 8% for each sample. Generally, three methods were in accordance with each other. So, FPIA was a method dependable and faithful for detection of authentic samples.

4. Conclusion

In summary, a rapid, sensitive and reliable immunoassay method (FPIA) was developed in this study. It demonstrated a high efficiency for detection of BPA with good monoclonal antibody and appropriate tracer. Moreover, FPIA had a good sensitivity with an IC_{50} value of 140 ng/mL and a limit detection of 5.60 ng/mL. It showed a negative cross-reactivity with various structural analogs except BVA. Then, the performance of the developed method in environmental water samples was investigated, with its recovery rate in sample range from 87.91%-114.28%. In addition, the detection data of FPIA in authentic samples were compared to those of ELISA and HPLC. These results indicated that the proposed FPIA could be suitable for detection of BPA in water samples.

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Table1 Cross-reactivity (CR) of FPIA for BPA and related compounds.

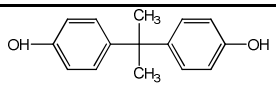
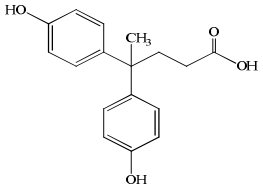
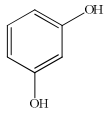
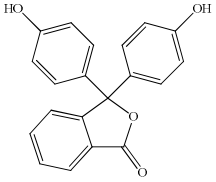
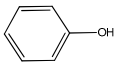
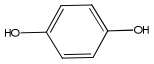
Compound	Structure	IC_{50}(ng/mL)	CR (%)
BPA		140.60	100.00
BVA		132.94	105.76
BPS		>100000	<0.15
Phenolphthalein		>100000	<0.15
Phenol		>100000	<0.15
Hydroquinone		>100000	<0.15
Benzene		>100000	<0.15

Table2 Analytical results and recoveries of four environmental water samples (n=3) with three immunoassay methods.

Sample	Theoretic concentration (ng/mL, n=3)	FPIA detected concentration (ng/mL, n=3)	Recovery (%)	ELISA detected concentration (ng/mL, n=3)	Recovery (%)	HPLC detected concentration (ng/mL, n=3)	Recovery (%)
Pearl river	10.00	9.71±0.40	97.10	9.26±0.24	92.60	8.54±0.12	85.40
	50.00	47.02±1.22	94.04	54.43±1.07	108.86	47.35±0.86	94.70
	100.00	87.91±6.68	87.91	110.16±4.65	110.16	95.97±3.22	95.97
Central lake	10.00	11.07±0.35	110.70	11.27±0.32	112.70	8.96±0.15	89.60
	50.00	57.14±2.17	114.28	55.02±0.98	110.04	47.68±1.07	95.36
	100.00	101.63±5.32	101.63	106.15±3.73	106.15	97.92±3.34	97.92
GDUT lake	10.00	9.60±0.62	96.00	10.85±0.41	108.50	10.21±0.36	102.10
	50.00	54.41±4.06	108.82	50.63±1.74	101.26	47.46±1.78	94.92
	100.00	112.17±5.01	112.17	109.72±3.79	109.72	97.17±4.01	97.17
Tap water	10.00	11.74±0.21	117.40	10.58±0.12	105.80	10.07±0.28	100.70
	50.00	53.63±1.98	107.26	51.76±0.73	103.52	49.27±0.72	98.54
	100.00	110.38±6.18	110.38	97.21±2.54	97.21	104.18±5.02	104.18

Pearl water: pearl river tributary;

Central lake: the water from the central lake in Guangzhou Higher Education Mega Center;

GDUT lake: the water from lake in Guangdong University of Technology;

Tap water: the water from our laboratory tap.

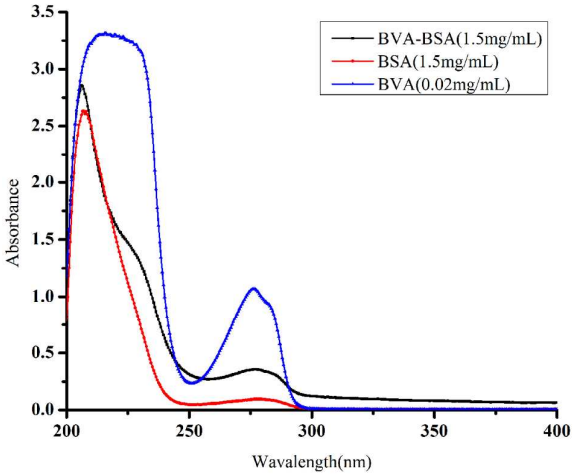


Fig.1 The UV absorbance spectrum of BVA, BSA and BVA-BSA solutions in PBS.

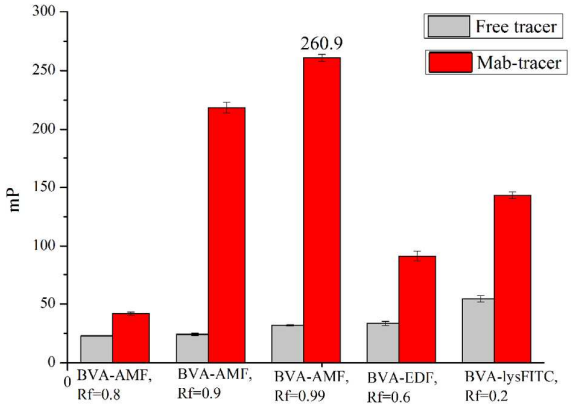


Fig.2 The binding of 5B8 MAb with BVA-AMF tracer in BB working solution.

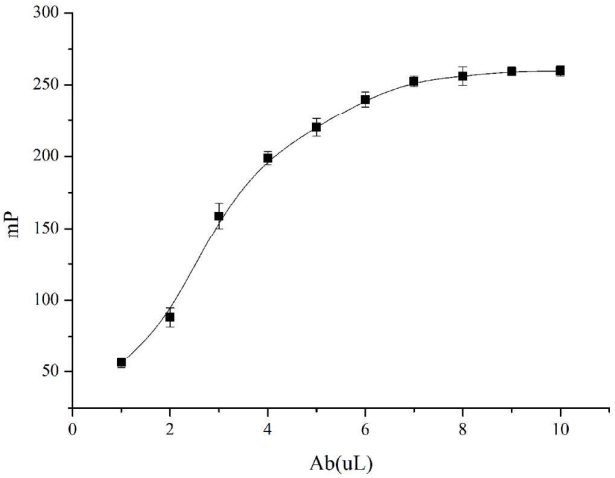


Fig.3 Dilution curve for 5B8 MAb with BVA-AMF tracer in BB working solution.

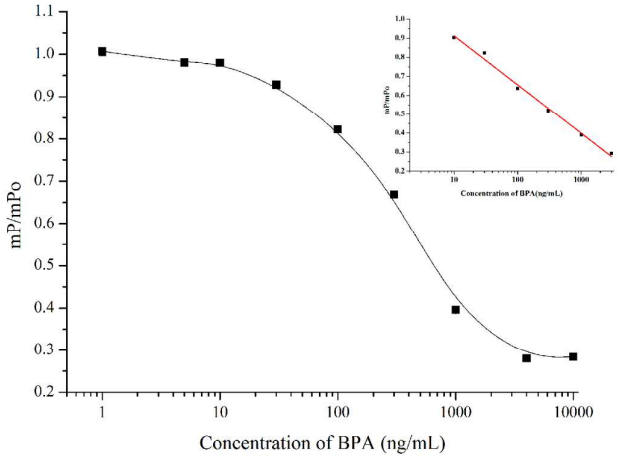


Fig.4 FPIA calibration curve and linear fitting for BPA using 5B8 MAb and BVA-AMF tracer.