Suspension array platform based on aptamer for high-throughput detection of five environmental hormones

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Methodology

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Abstract

Background

As a common small molecule substance, environmental hormones widely exist in nature, especially water sources, which have a profound effects in humans. Highly efficient and sensitive method for estrogens in the environment are essential.

Results

In this paper, a novel high-throughput platform was established based on five small hormones molecules specificity aptamer and magnetic beads (MBs). The results showed that the sensitivity of the proposed method are greatly improved. The limit of detection(LOD) of this method for atrazine(Atz), profenofos, bisphenolA(BPA), estradiol(E2), and polychlorinated biphenyls(PCBs) were 9.46, 20.75, 23.81, 8.97, 6.27 pg/mL, respectively. The Recovery rate of the diluted environmental hormones spiked in the samples of Haihe river were in the range of 87.5-111.02% with relative standard deviations (RSDs) lower than 28.44%.

Conclusion

This platform based on new complementary strand fragments can simultaneously rapid detection five environmental hormones. The whole procedure completed within 1.5h including sample treatment, incubation and detection, greatly improving the detection efficiency.

Backgroud

As a large family, environmental hormones are divided into several categories and widely exist in the environment and water. Most of environmental hormones are synthetic and used in industry. For example, BPA is widely found in industry which commonly used in mineral water bottles, coatings inside beverage tanks, mouth cups for baby and food products[1]. PCBs are additives to many industrial supplies[2]. Profenofos and atrazine are applied in agricultural as anti-helminths and herbicides[3, 4]. These environmental hormones will accumulate in livestock and crops with sewage and soil, finally enter the human body. Because of trace amounts can cause great harm to the human body[5, 6], such as infertility, neurotoxicity and low immunity[7, 8], environmental hormones have attracted wide attention of various governments[9, 10]. Therefore, it is particularly urgent to develop high sensitive, stability method to monitoring foodstuff and water environment .

Currently, classical methods for detect environmental hormones including colorimetry, enzyme linked immunosorbent assay (ELISA), and gas chromatography-mass spectrometry (GC/MS)[11–13]. Colorimetry can directly determine the concentration of environmental endocrine in the system through

the change of color, the disadvantage is that the quantification is not accurate enough. As a mature and common commercial immunofluorescence detection method, ELISA[14–17] could quantitative and qualitative detection environmental hormones. In recent years, various nanomaterials combination with ELISA and improved the sensitivity of method[18–20]. GC-MS is a very sensitive detection method which LOD can reach pg level. The disadvantage is instrument used in this method bulky and high requirement for operators. The simultaneous acquisition of multiple target signals in one sample is research hot topics, which is also absent from conventional detection methods. In recent years, aptamers combined with other technologies have been widely used in medical diagnosis[21, 22], food[23], and environmental monitoring[24–26]. As a recognition molecule, aptamers can replace antibodies for microorganisms[27, 28], cells[29], and heavy metal ions[30, 31]detection. Compared with antibodies, aptamers have the advantages of higher modifiability, variable structures, easy amplification which suitable for high-throughput detection. More important, aptamers are rapidly synthesized[32], inexpensive and ideal alternatives to antibodies.

Single-stranded oligonucleotide sequences are optimized and screened through exponential enrichment (SELEX), and obtain the most suitable aptamers for detection[33]. Suspension array technology (SAT) differs from ordinary solid-phase chips. Two different dyes were used in different proportions to obtain 100 different colors MBs. The flexible multiple-analyte system(xMAP®) can simultaneously detect 100 kinds of targets. The instrument can emit red light and green light at the same time, and the MBs can be analyzed quantitatively and qualitatively through these two kinds of light. At present, the suspension array technology is widely used in allergens[34], thrombin[35, 36],etc. These aspects are mostly combined with nucleic acid amplification (PCR, RCA) methods to improve the sensitivity of detection.

In this work, five environmental hormones were selected and coupled to the MBs of suspension array system for the first time, and designed biotin modified complementary chain (biotin-cDNA) in the semi binding region (Fig. 1). Finally, the environmental hormones were qualitatively and quantitatively analyzed by identifying the mean fluorescent intensity(MFI) of MBs through SAT platform. In our work, we innovatively designed biotin-cDNA in the half of the environmental hormones binding region, and the remaining design was in the adjacent region. The final results showed super sensitivity. We finally completed the assay for detection five environmental hormones in one hour. The application of aptamers, greatly reduced the cost of whole detection.

Materials And Methods

Chemicals and Reagents

Estradiol(E_2), bisphenol A(BPA) and [N-Morpholino] ethanesulfonic acid (MES) were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China). Polychlorinated biphenyls(PCBs), profenofos, atrazine(Atz) were purchased from Beijing Putian Tongchuang Biotechnology Co., Ltd. Different fluorescence-encoded MagPlex® MBs with carboxyl group were purchased from Luminex Corp. (Austin, TX, USA). Aptamer and biotin-cDNA purchased with Shanghai Shenggong Biological Company (Table S1)

and dilute to 10μM before use. Streptavidin phycoerythrin (SA-PE, 1 mg/mL) was supplied by Invitrogen Corp. (Carlsbad, CA, USA). 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride. (EDC), N-Hydroxy-sulfo-succinimide (S-NHS) were purchased from Pierce, USA. All reagents are analytical grade.

Apparatus

An American scanning electron microscopy(FEI Inspect F50, USA) was used for the characterization of the surface and section of the MBs. SAT (Luminex Corp., Austin, TX, USA) was employed for fluorescent signal processing. A 96-well plate with a black bottom and a transparent bottom is used to separate MBs from the solution. Zeta potentiometer (Malvern ZS90, UK) is used to verify whether the aptamer and biotin-cDNA are successfully coupled to the surface of the MBs. Use F-97 series fluorescence spectrophotometer to verify the feasibility of the experiment, Shanghai Lengguang Technology Co., Ltd. Using circular dichroism analyzer (JASCO J-715) to verify the binding ability of aptamers and small pesticide molecules.

Optimization of reaction conditions

Explore the optimal concentration of the aptamer for coupling, divide the aptamer into different concentrations, and couple it to the MBs. Add 1:100 SA-PE and react for 0.5 h. Wash the MBs, add 100μL sheath solution, and detect the MFI on the machine. Set different coupling times and coupling temperatures, and explore the effects of time and temperature on the results.

Divide the coupled MBs into four parts, and then add them to different storage buffers (HEPES buffer; 1xPBS buffer; hybridization buffer; Tris-EDTA buffer) overnight. Take 3 test tubes and add 20 μL of coupled MBs to each test tube. Add 10μL of small molecule standard solution and 4μL of biotin-cDNA to each test tube. React at 37°C for 1h. Then wash the MBs, add 1:100 SA-PE, and react for 0.5 h. Wash the MBs, add 100 μL of sheath fluid, and detect MFI on the machine. Take the concentration of the small molecule standard at 100ng/mL, add the small molecule standard and biotin-cDNA. Wash the completely reacted solution 3 times, add 1:50; 1:100; 1:200 SA-PE, react for 0.5h. Wash the MBs, add 100μL of sheath fluid, and detect MFI on the machine.

Process of the technology

Add 10μL of 5 kinds of aptamer-coupled MBs to each well of the washed black opaque 96-well plate. Then add 5 μL of small molecule standard solutions of different concentrations, react at 37°C for 0.5 h, after that add 4 μL of complementary strand to ensure final concentration ratio of aptamer to complementary strand is 1:1. Fill each well to 100 μL with coupling buffer. After magnetic separation, add 1:100 SA-PE 50 μL and react for 0.5 h, then magnetic separation, and read out the MFI on the machine.

Specific recognition

Five environmental hormones and their corresponding analogs were formulated into solutions with a concentration of 10 ng/mL. The 96-well plate was divided into five groups, and each group was divided into targets and their corresponding analogs. Add 10 μL aptamer-coupled microspheres to the wells, then

add 5 μL corresponding small molecules or the like, and incubate at 37°C for 0.5 hours. Then add the corresponding complementary strand and incubate for 0.5 hour in the same environment. After washing the plate, add SA-PE and react at 37°C for 0.5 hours, and final results were read out by machine.

Recovery tests in river samples

To confirm the availability of the assay, we prepared five small molecule standards solutions with a concentration of 1 and 10 ng/mL and add them to the Haihe river sample (the samples were collected in Haihe River, Tianjin, 117° 12' 7" E, 39° 06' 41" N). All the samples were filtered through 0.45 μm microfiltration membranes before test. Add 10 μL of 5 kinds of aptamer-coupled MBs to each well of the washed black opaque 96-well plate. Then add 5 μL of the spiked samples above. React at 37°C for 0.5 h, and then add 4 μL of biotin-cDNA to ensure that the final concentration ratio of aptamer to biotin-cDNA is 1:1. Each sample was measured three times, and the test values were calculated based on the standard curves, we also calculated the recovery and relative standard deviations (RSDs) of each sample.

Results And Discussion

Experimental feasibility verification

The five aptamers came from SELEX screening results, and we also designed five corresponding biotin-cDNA (all the sequences list in Table S1). A circular dichromatogram (CD) was used to verify the binding ability of small molecule standards and aptamers. It can be seen from the results that the small molecule standard has no obvious absorption peak, while the aptamer shows positive and negative peaks at 273nm and 245nm, respectively. When the aptamer is coupled with a small molecule standard, the peak amplitude will be significantly reduced, but the peak position will not change (Fig.S1). The combination of the aptamer and the small molecule standard will cause the conformational change of the aptamer, which leads to the change of CD amplitude.

The feasibility of this method is verified by fluorescence experiment. Take estradiol as an example (Fig.2). when E2 standard absence, the fluorescence value is the highest. After adding different concentrations of targets, the fluorescence value and the concentration of small molecule standards were negative correlated. This is because the concentration of the small molecule standard competes with the biotin-cDNA for the aptamer, and as the concentration of the standard increased, the biotin-cDNA bound to the aptamer decreases, resulting in a decrease of fluorescence value. The result conformed to the experimental principle and proved the feasibility of the experiment.

Optimization of reaction conditions

During the reaction, we evaluated several parameters to achieve the best fluorescence response. It can be seen from Fig.S2 that the difference of fluorescence value is not significant under different MBs stock solutions, but in 0.1M PBS buffer, the fluorescence value is the highest. Considering economy and

universality, we chose 0.1M PBS as stock solution. As a costly reporter molecule, the concentration of SA-PE need to be considered. The results showed that when SA-PE was diluted 1:50 and 1:100, the MFI change not significant considering the cost, we finally determined the optimal concentration of SA-PE was 1:100 (Fig.3a). By optimizing these conditions, the measured results can reach the optimal value. We also explored the optimization of coupling conditions and the results showed that the optimal coupling time was 1 h (Fig.S2), and the optimal coupling temperature was 37℃ (Fig. 3b).

After confirming that the aptamer was successfully coupled to the MBs, the optimal coupling amount of the five aptamers on the MBs was explored (Fig. 4). With the increase of aptamer concentration, MFI gradually increased and finally entered a smooth period or decreased. We select the point of maximum MFI as optimal coupling concentrations. The maximum coupling aptamer concentration for estradiol, atrazine, profenofos, polychlorinated biphenyls, and bisphenol A are 4, 2, 3, 5, 2μM respectively.

Characterization of surface modification of Aptamer-coupled MBs

The MBs, MB-Apt, MB-Apt-cDNA complex were characterized by scanning electron microscope (SEM) and Zeta potential. The SEM can enlarge targets tens of thousand times and intuitively observe the structural changes on the surface of substance. The zeta potential informs the degree of bonding between chemical bonds through the change of potential[37]. We employ SEM to scan the surface of MBs (Fig. 5). From Fig. 5a we can see that the blank MB is rough surface, when coupled with aptamers, the surface of the MBs is covered with a thin layer of protrusions (Fig 5b). Fig. 5c showed the surface of the MBs which were wrapped by biotin-cDNA and small molecule standards. We can see irregularly shaped protrusions on the surface of MB, indicating the surface of MBs covered by the complex.

We also characterized the zeta potential to observe of the potential change of coupling MB. Take the example of E2 coupling MB (Fig. 5d), we can see that carboxylate blank MB exhibits a zeta potential of 90.7±0.52mV, the value significantly drops to 22.9±0.41mV after coupling with aptamer, because amino aptamer coupling with carboxyl group on the MB and neutralized the potential. When add biotin-cDNA and form MB-Apt-biotin-cDNA complex, the zeta potential is 24.9±0.73mV. The result indicated the combination of biotin-cDNA and aptamer did not cause obvious potential change.

The specificity of assay for the detection of environmental hormone

Specificity is an important indicator to evaluate one assay. We selected two analogs for each of five environmental hormones, and the concentration was 10 ng/mL. When five targets were added to the detection system, the small molecules competed with the complementary chain for the corresponding aptamer, resulting in a decrease in MFI（Fig. 6）. When the analog exists in the detection system, the

analog cannot specifically bind to the aptamer, and the fluorescence value is not significantly reduced. It is proved that the five environmental estrogens have good specificity and can be used in real samples detection. The stability and sensitivity of this method are verified. In addition, during the detection process, only need two-step washing. We can complete the assay in about 1.5 h.

The sensitivity of assay for the detection of environmental hormones

After the conditions were optimized, different concentrations of small-molecule targets were added and the Logistic fitting equation was used to establish a single-channel detection. It can be seen that all of the five single-channel curves have an "S" shape. As the concentration of small molecule targets increased, the MFI gradually decreaseds, which is consistent with the experimental setup. By this method, the LOD of atrazine, profenofos, bisphenol A, estradiol, and PCBs were 8.5589, 16.8631, 23.2314, 8.4511, 6.2609 pg/mL(Table.1) respectively. The single-channel detection curve of these targets can be completed on a 96-well plate, and the detection time is between 40-45 minutes, which greatly shortened the detection time.

We obtained the optimal reaction conditions, and draw the multi-channel curve of five environmental endocrines, intuitively obtained the linear regression curves of five small molecule standards in SAT. The result showed a remarkable value, The LOD of Atz,

Profenofos, BPA, E2, PCBs reached 9.465, 20.7514, 23.8094, 8.9712, 6.2677 pg/mL, respectively (Table 2). However, the simultaneous addition of five small molecule standards in per well slightly affects the fitness of standard curve(Fig.7), resulting in not all the determination correlation coefficient (R^2) being greater than 0.98. Table 1 showed the Equation, LOD and R^2 of five small molecule standards.

We also compared this method with the classic ELISA experiment. The LOD of profenofos by ELISA was obtained experimentally, and the rest were cited in the literature. Through comparison, it can be seen that the detection limit of this method is lower than that of ELISA (Table 3), the LOD of this method was 20-400 times lower than that of ELISA.

Single-channel detection and multi-channel detection are the same for each type of small molecule coupled microspheres. Under the same coupling and detection conditions, the corresponding curves are obtained. Compared with single-channel detection, the number of probe microspheres contained in each well has been increased by five times, and the content of other substances in each well is also as high as a dozen, making multi-channel detection theoretically more difficult. However, duo to the fact that SAT allow all the substances to be detected in the liquid phase, and the sampling is more uniform, the result present a high specific result. Although the multi-channel detection system will be affected to some extent, it also forms a good linear relationship and a lower detection limit. Proved that the multi-channel detection system can be used for high-throughput and high-sensitivity detection.

Table1 Standard curves of five small molecule standards and related parameters determined by single-channel detection method

Item	MBs Code	Standard curve	R^2	LOD (pg/mL)
Atz	35#	$Y=200.03+2079.75/[1+(x/0.0022)^{0.24987}]$	0.9997	8.559
Profenofos	36#	$Y=140.58+1103.96/[1+(x/0.4601)^{0.5241}]$	0.9942	16.86
BPA	42#	$Y=1351.23+5007.24/[1+(x/0.918)^{0.31376}]$	0.9971	23.23
E_2	45#	$Y=481.45+1079.14/[1+(x/0.018)^{0.31246}]$	0.9945	8.451
PCBs	54#	$Y=518.29+4913.32/[1+(x/6.6819)^{0.35056}]$	0.9926	6.261

Table 2 Standard curves of five small molecule standards and related parameters determined by multi-channel detection method

Item	MBs Code	Standard curve	R^2	LOD (pg/mL)
Atz	35#	$Y=0.12177+1.06589/[1+(x/0.67263)^{0.5932}]$	0.9998	9.465
Profenofos	36#	$Y=0.14602+1.51776/[1+(x/0.00221)^{0.2499}]$	0.9978	20.75
BPA	42#	$Y=-0.22115+0.81952/[1+(x/0.91874)^{0.3732}]$	0.9942	23.81
E_2	45#	$Y=0.37035+0.83011/[1+(x/0.01837)^{0.3124}]$	0.9893	8.971
PCBs	54#	$Y=0.19544+1.46436/[1+(x/9.7887E\text{-}5)^{0.5782}]$	0.9856	6.268

Table 3 Comparison of this method with classic ELISA

Item	This Work(pg/mL)	ELISA(ng/mL)
Atrazine	9.4653	0.500[38]
PCBs	6.2677	0.020[37]
BPA	23.8094	0.200[37]
Profenofos	20.7514	0.150
E_2	8.9712	0.020[37]

Spiked actual samples

To investigate the ability of the method in practical application, we selected water from the Haihe River to carry out a spiked recovery test. The relative standard deviation of the actual sample detection is between 7.64-28.44%, and the relative standard deviation of estradiol is relatively high. In the case of high-throughput detection, all meet the detection standards (Table 4). It can be seen that this method can be used for the detection of estradiol, bisphenol A, polychlorinated biphenyls, atrazine, and profenofos in water. Of course, these five targets can also be randomly matched for actual sample detection. The test can be performed on the premise of setting the method, and the detection of samples it only takes about 1 h to complete, and the number of MBs read in each sample well (usually 50 or 100) can be set. This detection is rapid and accurate, and has great practical application value.

Table 4 Determination of five small molecule standards in the environmental water samples by the novel SA.

Item	Actual concentration(ng/mL)	Detect concentration(ng/mL)	RSD (%)
Atz	1.00	0.875	17.35
Profenofos	10.0	11.10	8.060
PBCs	1.00	0.956	14.91
E_2	10.0	11.01	28.44
BPA	10.0	9.906	7.640

Conclusions

Rapid detection techniques for food and water environmental contaminants are an hot topic of research. This is because toxins, endocrine disruptors, heavy metals present in nature will enter the human body through water sources, food, and trace concentrations will have a profound effect on the human body[39]. Despite the traditional monitoring methods playing a great role in daily detection, not fast enough[40], single-thoughput[41] are still the limiting factor that needs to be addressed. As a detection platform with mature applications in medicine, the suspension array technology has high sensitivity and specificity, which can be applied as a promising technology in the rapid detection of water environment and food products.

In short, we selected a new fragment complementary to the aptamer, and indirectly competed with the environmental hormones for the aptamer through the biotin-cDNA, and completed the high-throughput detection of five targets. The selected new complementary fragments which modified with biotin improved the sensitivity of detection. It has been demonstrated that the unmodified aptamer reduced the cost of detection and increased the flexibility of detection. Compared with the previous report[42], the sensitivity of five small molecules is greatly improved and realized high-throughput detection.

In this study, we have demonstrated the great advantages of using aptamers instead of antibodies as probes for monitoring small molecules of environmental estrogen. A standard curve of the assay was constructed and analyzed. Small molecule commercial antibodies are expensive, hard to obtain, and the synthesis of short aptamer chains is convenient, quick, and inexpensive. The results also showed that the aptamer and small molecule had a good binding force, and the detection limit reached the pg level.

The SAT is green and simple. The assay can be performed in a two-step process utilizing a 96 well plate during the sample incubation stage and does not require special treatment by professionals. High throughput is undoubtedly a great advantage over conventional detection methods. The simultaneous detection of dozens of samples within 1.5h, including all the incubation, detection stages, greatly accelerates the progress and time.

In the field of rapid detection, it is only of practical interest to be able to apply the method to on-site monitoring. The good or bad of water quality directly relates to human health. We selected Haihe Water, which is closely related to living, as a sample, and performed a spike recovery test, finally the satisfactory results were obtained. At different spiked concentrations, the final recoveries rate were all between 87.5-111%, indicating that the established platform can be applied in real sample detection.

In summary, the combination of aptamers with SAT enables rapid, high-throughput, sensitive detection using minimal sample volumes, green and label free. This novel platform can be used as a tool to detect and establish small molecule solutions to environmental pollution. In addition, this system serves as a research tool for pollutant changes in the environment. Further technological development as well as advanced studies have shown that the system can be integrated into food and water environments for monitoring contaminant traces to prevent hazards to living organisms.

Declarations

Supporting Information

We list the method used in our experimental. Figure S1 showed The CD chromatogram of five environmental endocrine, Figure S2. The optimization of coupling conditions. Table S1 is aptamers selection of five environmental hormones and their complementary chains

Authors' contributions

All authors contributed to the experimental procedures and the writing of this manuscript. All authors approved the final version of the manuscript.

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Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Figures

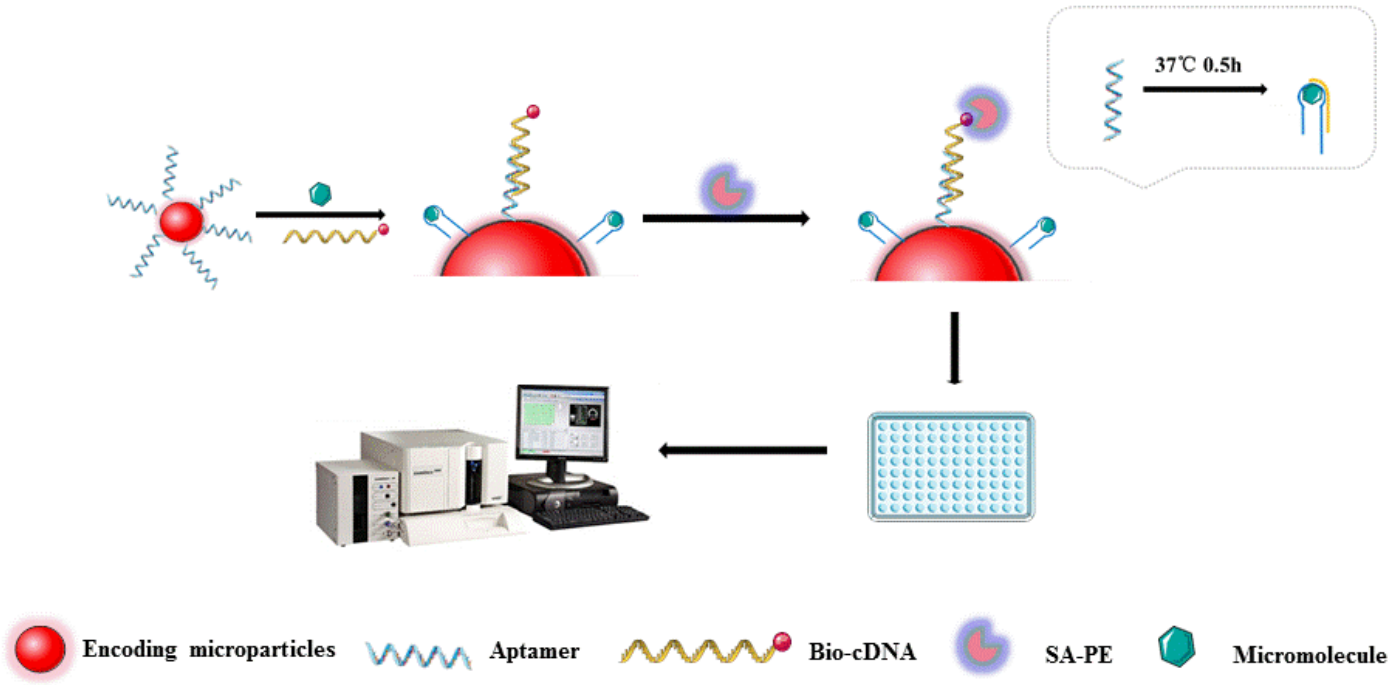

Figure 1

Schematic diagram of the indirect competition SAT for environmental hormones with complementary chains for high-throughput detection.

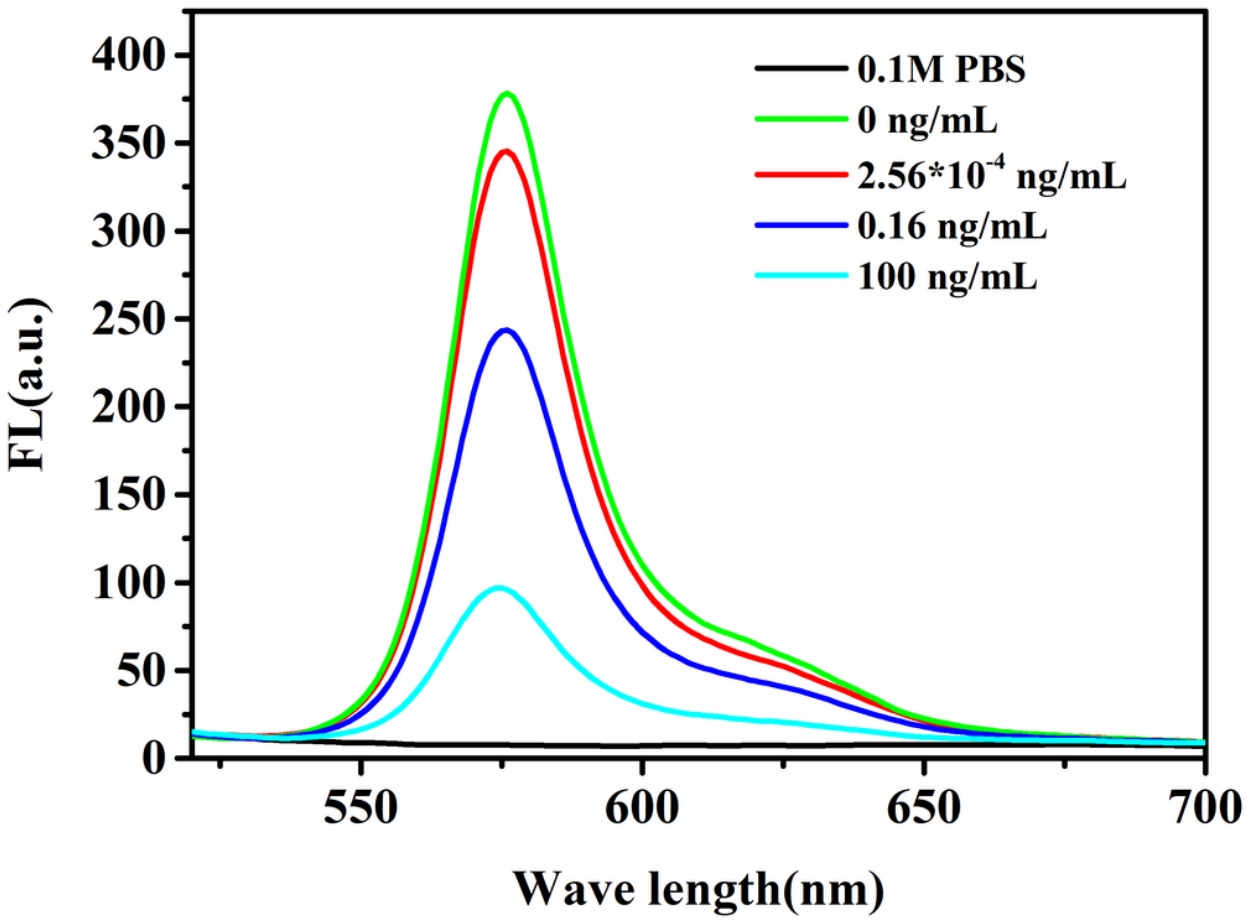

Figure 2

FL response under different concentrations of E2

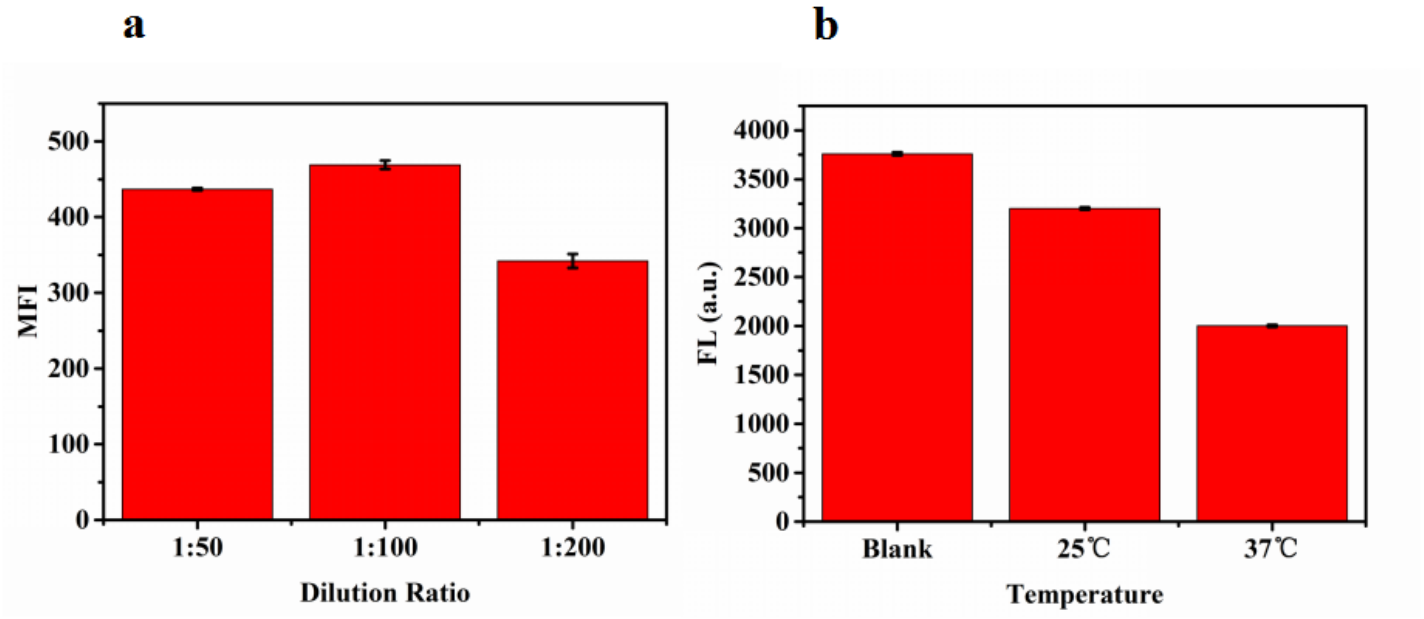

Figure 3

The optimization for (a) SA-PE dilution ratio, (b) Incubation temperature. The error bars represented the relative standard deviation of the three groups of paralleled measurements

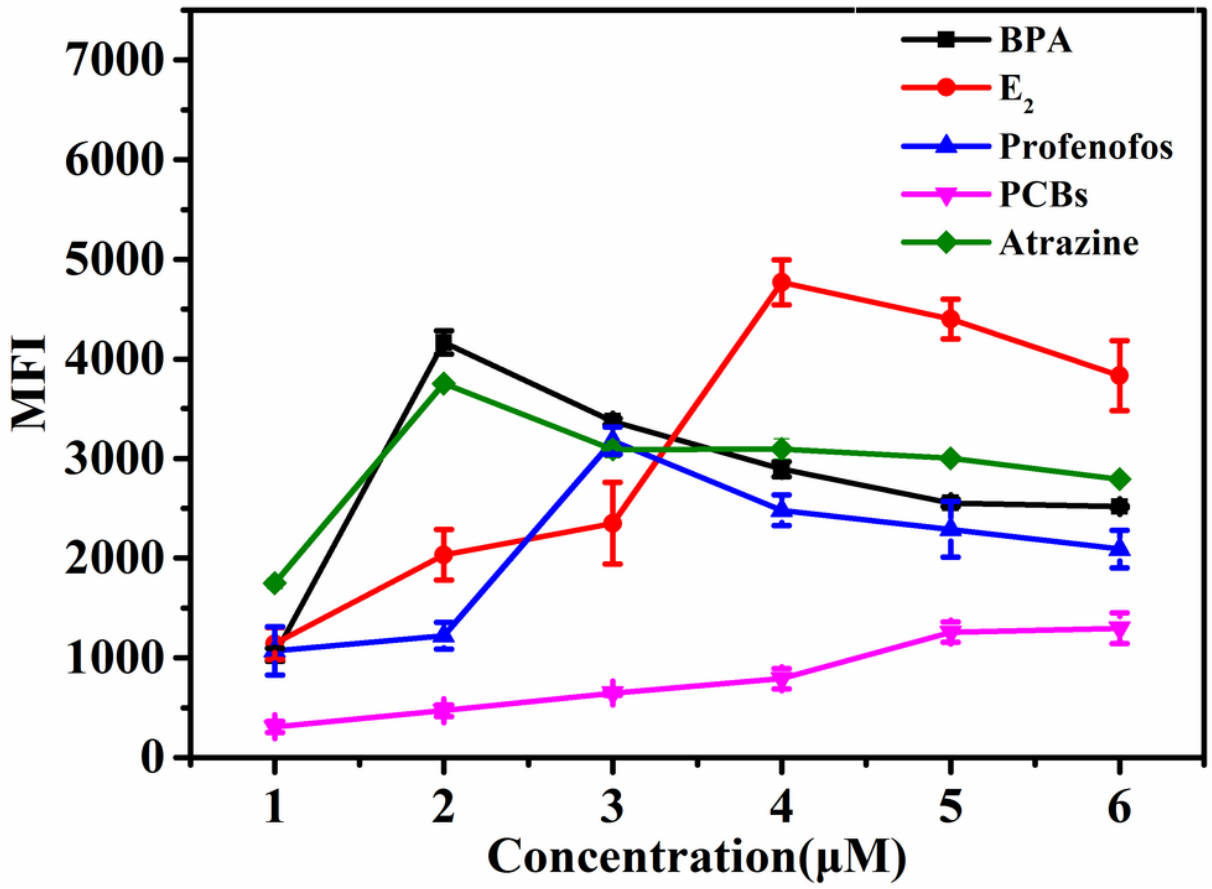

Figure 4

The optimization for coupling amount of aptamer. The error bars represented the relative standard deviation of the three groups of paralleled measurements

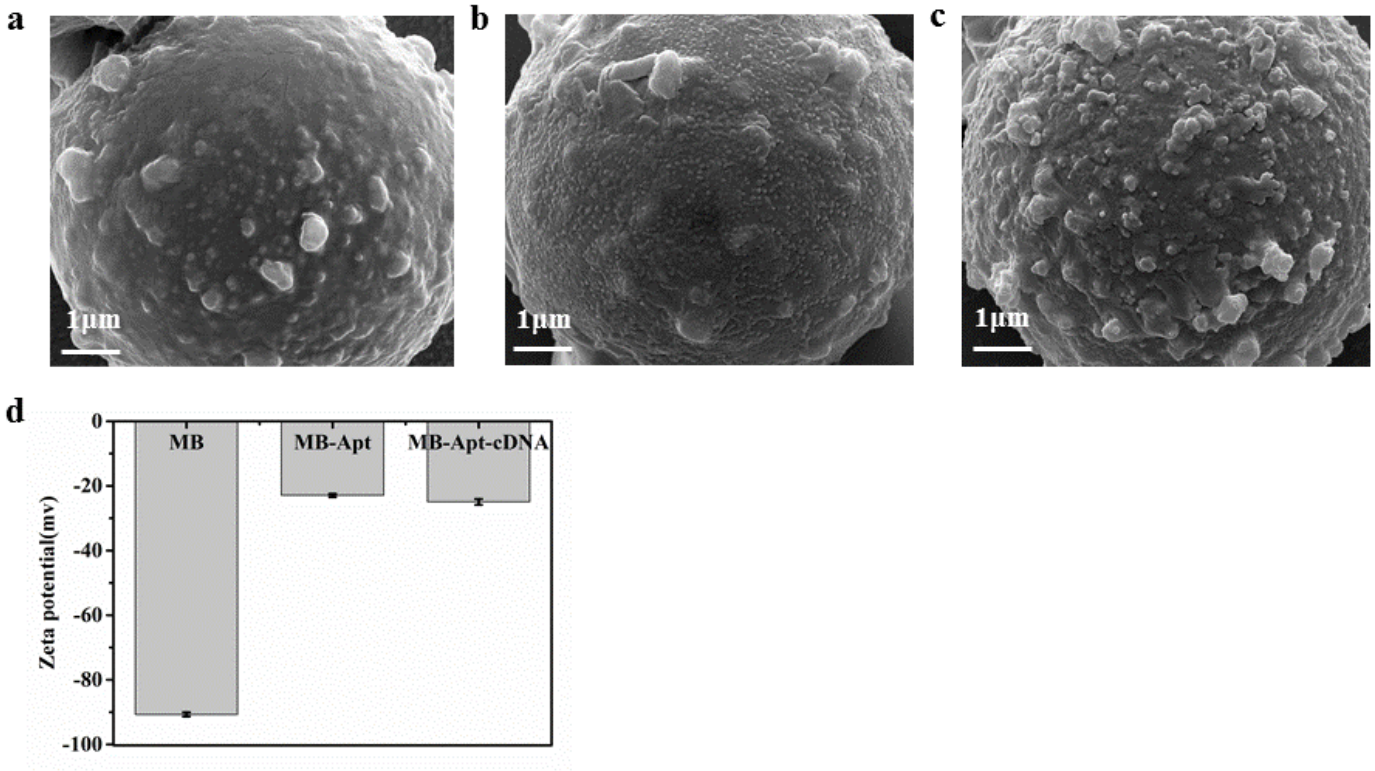

Figure 5

Monitoring of the binding and dissociation of biotin-cDNA and Apt from MB by zeta potentials and SEM. (a-c) SEM images of surface-coupled aptamer MBs (inset is the enlargement), the single MB-Apt-biotin-cDNA complex, and the single MB-Apt-cDNA with E2 complex. (d) The zeta potential monitoring of the MB, MB-Apt, and MB -Apt-cDNA.

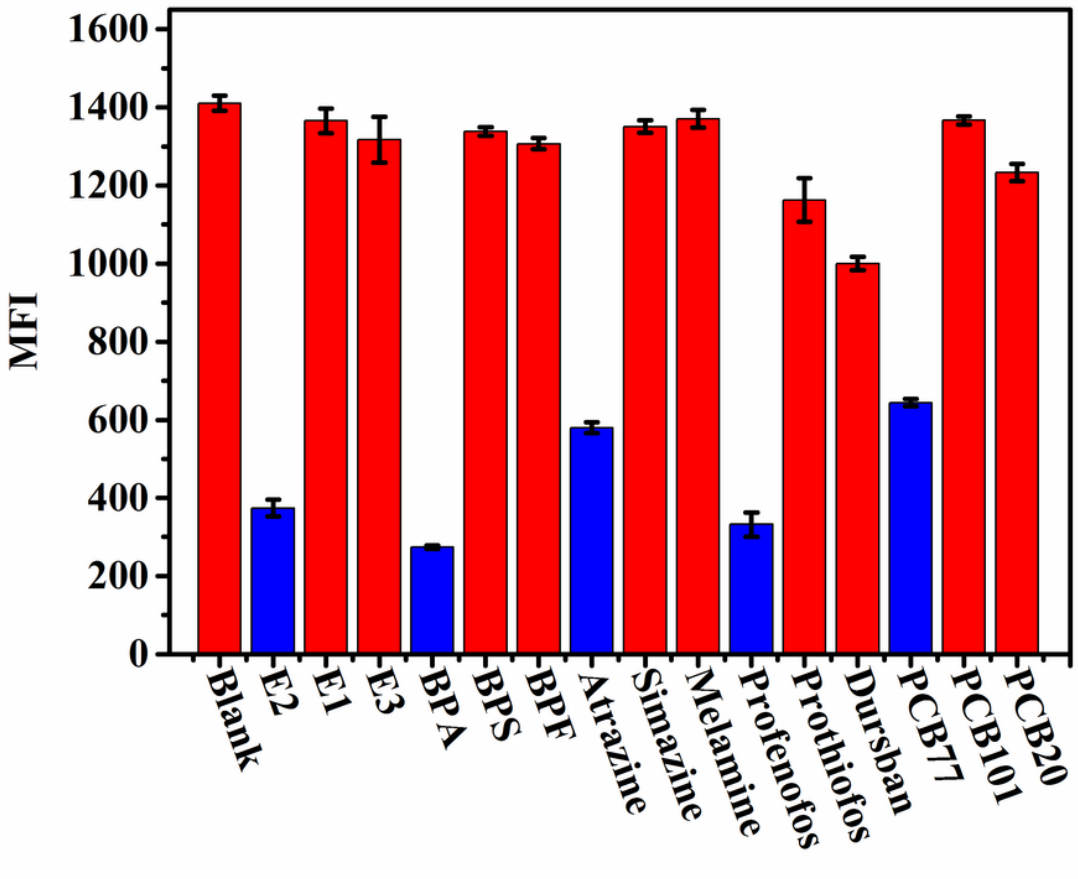

Figure 6

The Selectivity for possible interferences

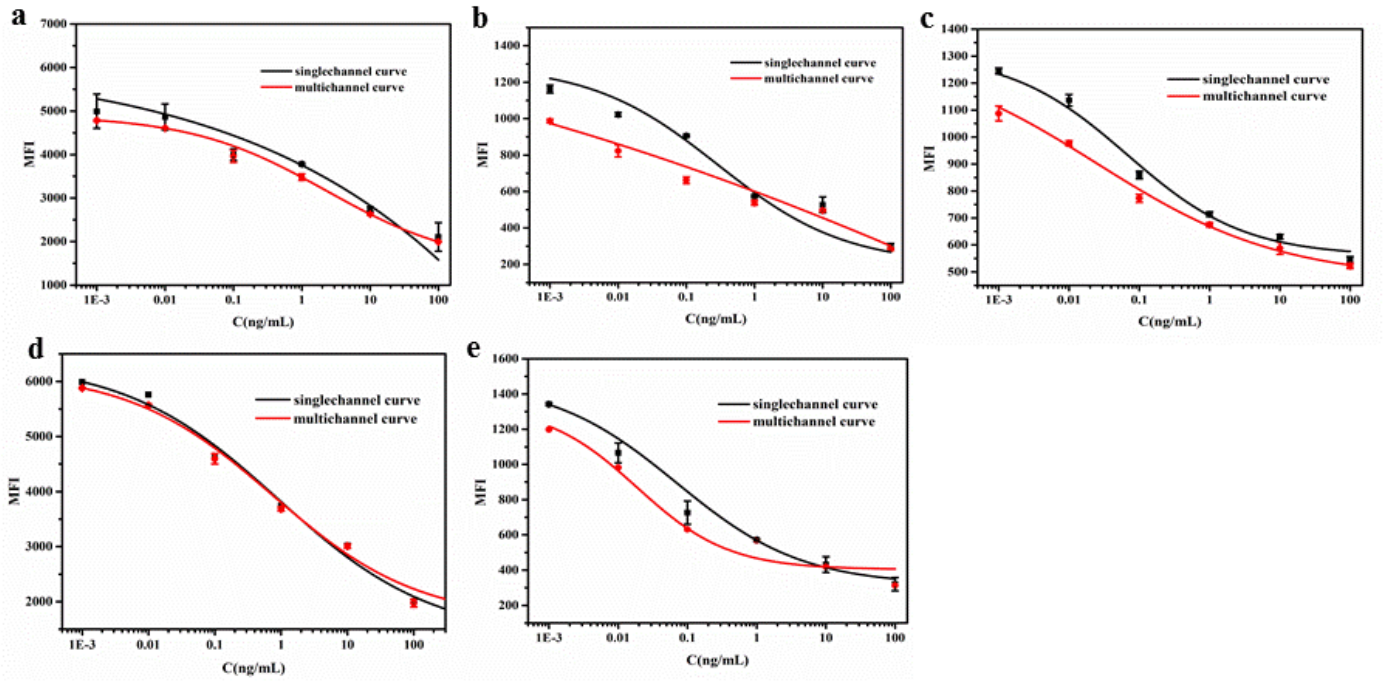

Figure 7

Comparison of single-channel and multi-channel curves of five environmental estrogen.(a) PCBs; (b) Profences; (c) E2; (d) BPA; (e)Atrazine

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