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Sensitive bioassay for detection of PPARα potentially hazardous ligands with gold nanoparticle probe

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ABSTRACT

There are so many kinds of peroxisome proliferator-activated receptor α (PPARα) ligands with hazardous effect for human health in the environment, such as certain herbicides, plasticizers and drugs. Among these agonists, perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), and mono-(2-ethylhexyl) phthalate (MEHP) are mostly investigated due to their persistence and accumulation in environment and their potential toxicity via PPARα. This investigation aims at developing a bioassay method to detect PPARα ligands based on the ligand–receptor interaction on microplate. PPARα, which formed heterodimers with retinoid X receptor-α (RXRα), were activated by PPARα ligands to form ligands–PPARα–RXRα complexes. Then the complexes were transferred into a microplate and captured via monoclonal anti-PPARα antibody. The PPARα responsive elements (PPRE) modified-gold nanoparticle probes were captured by the ligand–PPARα–RXRα complexes immobilized on the microplate, and then could be quantified through measuring the optical density after silver enhancement. The results showed that PFOS was quantified with a linear range from 100 pM to 1 μM and the detection limit was 10 pM. In addition to PFOS, PFOA and MEHP were also quantified within a proper range through the proposed bioassay. This bioassay was compared with that of liquid chromatography tandem-mass spectrometry (LC–MS) for water spiked samples with a significant correlation ($r = 0.9893$). This study provides a high-throughput detection method for PPARα ligands in microplate with high sensitivity and wide linear range. It may serve as an assistant of LC–MS for prescreening of PPARα ligands like PFOS.

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1. Introduction

Peroxisome proliferator-activated receptor α (PPARα) ligands include plasticizers, herbicides, endogenous steroids, hypolipidemic drugs, and solvents [1,2]. As a member of PPARα agonists, perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), and mono-(2-ethylhexyl) phthalate (MEHP) have been widely used in industry as surfactants, coating on fabrics, and so on; they have been detected in environmental waters, in wildlife and in humans [3–5]. As a class of global pollutants, now they are intensively studied by their bioaccumulation, biomagnification and toxicity mediated by peroxisome proliferator-activated receptor α (PPARα) [1,6,7]. In particular, PFOS and PFOA are pointed out to be persistent in the environment and appear to undergo no further degradation or transformation [8,9]. The current detection and quantification of these compounds mainly depends on liquid chromatography–tandem mass spectrometry (LC–MS–MS) [10,11], liquid chromatography–mass spectrometry (LC–MS) [12–14] and gas chromatography–mass spectrometry (GC–MS) [10], which are powerful, highly sensitive tools that allow determination of environmental concentrations of these compounds. However, these methods can only provide an accurate measurement of each of the known chemicals in sample extracts; it is unfavorable for high-throughput screening of large quantities of samples and not reliable for predicting the toxicity of complex mixtures of PPARα agonists. Hence, a rapid and simple analytical method is desired for the screening of these compounds.

In the present work, a method for determination and screening of PPARα ligands was developed, based on the ligand–receptor interaction [15]. PPARα is a ligand-dependent transcription factor that activates target genes [1,2,16]. It is revealed that PPARα

Abbreviations: GNP, gold nanoparticle; LC–MS, liquid chromatography/tandem mass spectrometry; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; MEHP, mono-(2-ethylhexyl) phthalate; PPARα, peroxisome proliferator-activated receptor α; PPRE, PPARα-responsive elements; RXRα, heterodimers with retinoid X receptor-α.

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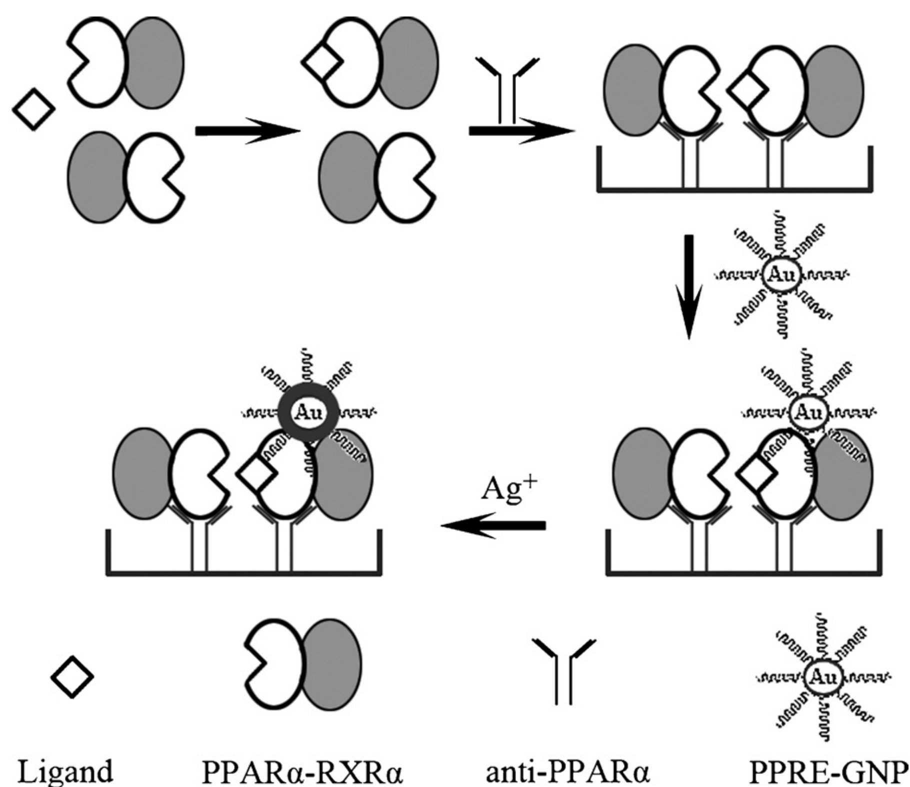

Scheme 1. Detection of PFOS based on the interaction between PPRE-modified gold nanoparticle probes and activated PPARα. PPRE can only be captured by activated PPARα. The reaction is displayed by silver enhancement. (PPRE–GNP, PPRE-modified gold nanoparticle probes.)

agonists like PFOS were associated with carcinogenesis through the peroxisome proliferative responses via PPARα [17,18]. PPARα and retinoid X receptor-α (RXRα) heterodimerize in vivo, with or without the presence of PPARα ligands, but the PPARα-RXRα heterodimerizers could hardly interact with peroxisome proliferator-response elements (PPREs) until it is activated by the ligands of PPARα [1,2]. That is, only when PPARα is activated by its ligands, it would interact with PPREs located in the promoter regions of target genes, and thus modulate the subsequent expression of target genes [19]. The binding of ligand, receptor, and response element can provide a cell free bioassay system for quantification and screening of receptor ligands [3,15], which may have the potential advantages of evaluation of the toxicity and potency value of PPARα ligands.

The recently developed bioassay based on oligonucleotide modified-gold nanoparticle probes has exhibited advantages in detecting a variety of bimolecular targets, including nucleic acids and proteins [20–24]. The gold nanoparticle probes have several advantages including nonradioactivity, high electron density and excellent biocompatibility [21,22,25]. Especially, the technique to use silver enhancement on captured gold nanoparticle probes can extraordinarily increase assay sensitivity [15,23,26].

Based on the interaction between PPARα and PPRE activated by the ligand, the present bioassay system was described as shown in Scheme 1. PPARα ligands firstly initiated the formation of ligand–PPARα–RXRα complexes, which were then transferred into a microplate and captured via monoclonal anti-PPARα antibody linkage. The PPRE modified-gold nanoparticle probes were added into the microplate and captured by the ligand–PPARα–RXRα complexes. Subsequently, a silver enhancement step was applied for signal amplification because of the polymerizing properties of gold nanoparticles to Ag particles. With the signal magnification by silver enhancement, the results could be recorded more easily (the sensitivity could be increased). Since PPRE could only be recognized by activated PPARα–RXRα complexes in the reaction system, the quantity of PPRE modified-gold nanoparticle probes captured by the ligand–PPARα–RXRα complexes would be proportional to the quantity of the ligands. Other ligands of PPARα could also be indirectly quantified by densitometry theoretically.

2. Materials and methods

2.1. Materials

All high-performance liquid chromatography (HPLC) purified oligonucleotide sequences were synthesized by Sangon Biotechnology Inc. (Shanghai, China). $AgNO_3$ and hydroquinone were also purchased from Sangon Biotechnology Inc. (Shanghai, China). PFOS, PFOA, and MEHP were purchased from Fluka (Buchs, Switzerland). Monoclonal anti-PPARα, Poly dI-dC and gold nanoparticles (15 nm) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Polyclonal anti-PPARα from rabbit (SC-9000) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All reagents for nuclear extraction were purchased from Roche Ltd. (Mannheim, Germany). Ultra-pure water (18 MΩ, Barnstead International) was used to prepare all the solutions if necessary.

2.2. Preparation of PPARα and Western blot

The nuclear receptors PPARα and RXRα applied in this assay were from the liver of male Sprague Dawley rats [27,28] according to the protocol of nuclear extraction described by Crinelli et al. [29]. Briefly, liver cells were separated and extensively washed with cold phosphate-buffered saline and lysed with cold buffer A [10 mM HEPES–KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.2 mM EDTA, 10 μg/mL Aprotinin, 10 μg/mL Leupeptin, 10 μg/mL Pepstatin, 0.5 mM Phenylmethyl Sulfonylfluoride (PMSF), and 0.6% Nonidet-P40]. The cell suspension was then chilled on ice for

15 min before centrifugation at 10,000 × g. The resultant nuclear pellet was resuspended in cold buffer B (20 mM HEPES–KOH, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 mM EDTA, 10 μg/mL Aprotinin, 10 μg/mL Leupeptin, 10 μg/mL Pepstatin, 1 mM PMSF) and incubated on ice for 30 min before being centrifuged at 15,000 × g. Nuclear extract supernatant was collected and stored at −80 °C.

The presence of PPARα in the extract product was identified by Western blot. Western blot analysis was done as the following steps. Protein concentrations in the supernatant were determined by BCA Protein Assay Kit (Beyotime, Shanghai, China). Due to the high concentration of the nuclear extract, it was diluted 1:10, 1:20 and 1:30, respectively, by buffer C (20 mM HEPES–KOH, pH 7.9, 25% glycerol, 50 mM KCl, 1 mM DTT, 0.2 mM EDTA and 1 mM PMSF) for the subsequent experiment. Proteins were electrophoretically resolved on 10% SDS-polyacrylamide gels, and then transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Membranes were incubated with specific primary antibodies (1:1000 diluted, Sigma–Aldrich Co. St. Louis, MO, USA), washed 3× with PBS for 15 min, and incubated with the horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:1000; Beyotime, Shanghai, China) for 1 h. The membranes were washed 4 times and developed with BeyoECL Plus (Beyotime, Shanghai, China). β-Actin was used as the internal control. Finally, the 1:10 diluted nuclear extract was adopted in the following experiment.

2.3. Preparation of PPRE modified-gold nanoparticle probes

The PPRE sequences were designed according to a previous report [16], shown in Table 1. The corresponding cold probe (without alkane-thiol modification) and mutant probe sequences were also designed to confirm the specificity.

Both of the PPRE and nonspecific competing probes were annealed under the following conditions: synthetic single-stranded probes were dissolved in sterile TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Each pair of probes were melted in annealing buffer (10 mM Tris–HCl, 1 mM EDTA, 50 mM NaCl, pH 8.0) at 72 °C for 10 min, followed by cooling to 45 °C for 1 h and then to 25 °C in a thermal cycler [30,31].

The oligonucleotide modified-gold nanoparticle probes were prepared as described. Briefly, 5 μL 100 μM PPREs (5 μM, final concentration) were initially incubated with 0.5 mL 10 nM gold nanoparticles resuspended in 95 μL phosphate buffered saline (PBS, pH 7.0, 50 mM PB, 0.10 M NaCl) for more than 16 h, followed by successive additions of 2 M PBS, at 6 h intervals, to a final concentration of 0.3 M NaCl. After an overnight incubation, the probes were isolated by centrifugation, washed in 100 μL water, and resuspended in a 50 μL 10 mM phosphate buffer (pH 7.0, 0.3 M NaCl, 0.01% sodium azide buffer). The PPRE modified-gold nanoparticle probes were stored at 4 °C. To minimize the effect of nonspecific binding in the subsequent assay, the probe-modified gold nanoparticles were incubated with 1% (w/v) BSA [32] and washed by 0.1 M PBS (pH 7.0) in advance. After that, 100 nM (final concentration) cysteine and glutamine (cys/glu) were mixed with nanoparticle probes to block the remaining binding sites on the surface of the gold nanoparticles which were not covered by BSA.

Table 1
Oligonucleotide sequences for probes.

Probe	Sequence
Alkane-thiol PPRE	5′-CAAATGTAGGTAATAGTTCAAAATAAAAAA-3′-(CH2)$_3$-SH 5′-TGAACTATTACCTACATTTG-3′
Cold PPRE	5′-CAAATGTAGGTAATAGTTCAAAATAAAAAA-3′ 5′-TGAACTATTACCTACATTTG-3′
Mutant PPRE	5′-GTCGTGCGTCTATGCTTGGAAAATAAAAAA-3′-(CH2)$_3$-SH 5′-TCCAAGCATAGACGCACGAC-3′

The Cold PPRE indicates that the recognition and binding property with the ligand–PPARα–RXRα complex has the same sequence as alkane-thiol PPRE, but it cannot conjugate with gold nanoparticles due to the absence of alkane-thiol modification, so it would not induce silver enhancement.
Mutant PPRE indicates that the sequence is different from PPRE, so it cannot be recognized by the ligand–PPARα–RXRα complex. This probe would not induce silver enhancement, because it cannot be recognized or captured by the PFOS:PPARα:RXRα complex, according to previous literature [16].

2.4. Standard solution and water spiked samples preparation

Serial concentrations of PFOS, PFOA and MEHP between 1 pM and 3 μM were prepared as standard solutions, and 0.05% dimethyl sulfoxide (DMSO) was used as a negative control. Ultra-pure water was used as PPARα ligands free water to prepare PFOS spiked water samples.

2.5. Environmental water samples pretreatment

Water samples were collected from Yangtze River in March 2010. The temperature, pH, turbidity and conductivity of water samples were measured, respectively, as 13.2 °C, 7.8, 13.8 mg/L and 397 μS/cm. Then water samples were transported to laboratory and pretreated by solid phase extraction. Approximately 1000 mL water was filtered through glass fiber filters (0.22 μm). The particulate matter collected on the filters was dried and then extracted with 5 mL methanol. The extraction solution was added to the 1000 mL filtered water. Subsequently, the water was loaded at a flow rate of 1 mL/min on a vacuum manifold onto a 600 mg C18 solid phase extraction cartridges (Oasis HLB extraction column 2.1 mm × 20 mm, 25 μm). Before the loading of the samples, the cartridges were preconditioned with 10 mL of methanol and 10 mL of ultra-pure water. Samples were eluted from the cartridge with 5 mL of methanol and collected in graduated glass tubes. The eluent was then concentrated to 1 mL under nitrogen. 500 μL aliquot of extract was transferred to a 1.5 mL glass auto sampler vial.

2.6. Detection of PFOS with PPRE modified-gold nanoparticle probes

100 μL 2.0 mg/L (final concentration) monoclonal anti-PPARα in coating buffer (Na_2CO_3 0.16 g, $NaHCO_3$ 0.29 g, 100 mL H_2O, pH 9.6) was immobilized in each well of a microplate overnight at 4 °C. After the wells were washed 2 times by 0.1 M phosphate buffer saline (PBS), 3% BSA was added into the well and incubated for 2 h at 37 °C for blocking of the free sites. In addition, an increased serial concentration of PFOS of 0.1 pM, 1 pM, 10 pM and 100 pM with 0.05% dimethyl sulfoxide (DMSO) as a negative control was incubated with the 1:10 diluted nuclear extract together for 2 h at 37 °C to activate PPARα. Then, 100 μL of the PFOS–PPARα–RXRα complexes were incubated with the antibody anti-PPARα coated in the wells for 2 h in order to be captured by anti-PPARα. Finally, the wells were washed by PBS buffer with 0.1% (v/v) Tween-20 for 3 min (three times).

After the ligand–receptor complexes were immobilized in the microwells, PPRE modified-gold nanoparticle probes (mixed with final concentration 1 μM poly (dI-dC) for minimization nonspecific receptor–probe interaction), which were expected to be specifically captured by ligand activated PPARα, were incubated with the PFOS–PPARα–RXRα complexes in reaction buffer (50 mM Tris–Cl, pH 7.4, 50 mM KCl, 25 mM $MgCl_2$, and 20% glycerol) for 30 min. In order to eliminate the interference of Cl^- with $AgNO_3$ in the subsequent coloration reaction, the wells were washed twice with PBS and PBN (phosphate buffered-sodium nitrate, without Cl^-), respectively. Subsequently, 100 μl silver enhancing solution was added to each well simultaneously with an Eppendorf multi-channel

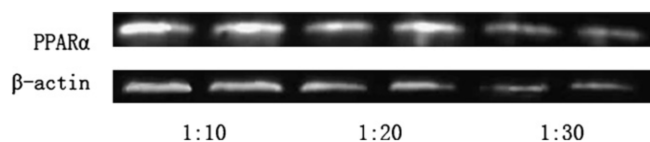

Fig. 1. Western blot of the PPARα from nuclear extract product.

pipette. Silver enhancing solution was $AgNO_3$ dissolved in citrate acid/trisodium citrate and hydroquinone [30,33] (0.5 g $AgNO_3$/2 mL H_2O, 1.7 g hydroquinone/30 mL H_2O and 2.55 g citric acid/2.35 g trisodium citrate/10 mL H_2O, mixed simultaneously) and incubated at 25 °C for 4 min. Finally, the wells were washed by ultra-pure water (18 MΩ) for 3 min (three times) and then the absorbance was detected. The optical density, which was consistent with the concentrations of PFOS, was recorded by a microplate reader. In this procedure, protection of the silver enhancing solution from light was recommended.

2.7. Detection of PFOS with LC–MS

LC–MS (Agilent 1100 Series LC–MS Ion-Trap, Agilent, Palo Alto, CA) was performed with a sample volume of 5 μL. Separation was achieved on an Inertsil ODS-3 column (2.1 mm × 150 mm, 5 μm; GL Sciences Inc., Tokyo, Japan). The column oven was maintained at 37 °C. Separation was carried out using a mobile phase of 1.0 mM ammonium acetate/methanol (v/v) at a flow rate of 0.2 mL/min. The valve was switched 11.5 min after sample injection. The gradient profile was as follows: linear increase from 40% to 75% methanol solution for 5–12 min, then hold at 75% for 3 min. The conditions for MS were as follows: desolvation and source temperatures were set at 325 °C and 100 °C, respectively, and the capillary was held at a potential of 1000 V relative to the counter electrode in the negative-ion mode for all compounds. PFOS and PFOA compounds are identified and further quantitatively analyzed by single mass mode using characteristic ions at m/z 498.9 and 412.9 for the determination of PFOS and PFOA, respectively. In order to obtain the calibration curves of PFOS and PFOA, the recovery rate for determination of PFOS and PFOA were evaluated by extracting blank spiked with standard 0.4 pM, respectively.

2.8. Statistical analysis

Linear regression was used to determine the relationship between detection signal and the concentration of PPARα ligands in the samples. The consistency of detection results from the bioassay and LC–MS for different concentrations of PFOS was analyzed by correlation analysis. *T*-test was used to determine the significance of differences between the results produced by the two methods for PFOS spiked water. Significance was accepted for p values < 0.05.

3. Results

3.1. Verification of PPARα in nuclear extract product

In order to investigate whether PPARα protein had been successfully extracted from liver cells, the nuclear extract was diluted to 1:10, 1:20 and 1:30 for Western blot confirmation. As shown in Fig. 1, the density of protein PPARα from nuclear extract product by diluting with 1:10 was higher than that of 1:20 and 1:30.

3.2. Reduction of nonspecific binding

Owing to PPRE modified-gold nanoparticles probe may nonspecific binding to protein in the microwells, two kinds of reduction of nonspecific binding were checked. One is with BSA incubated with PPRE modified-gold nanoparticles only. The other is with BSA, then the addition of cys/glu and poly (dI-dC). The optical density results after silver enhancement for different concentrations of PFOS are shown in Fig. 2. As expected, compared to with BSA only, the combination with BSA, cys/glu and poly (dI-dC) efficiently minimized the nonspecific binding, which reduced the background signals and provided a wider linear response for PFOS.

Table 2
Intra-day assay and inter-day assay imprecision for the PFOS test with PPRE-modified gold nanoparticle probes assay, indicated by the coefficients of variation (CV).

Concentration group (pM)	Intra-day assay CV (%)[a]	Inter-day assay CV (%)[b]
5.00	4.8	5.5
50.00	6.2	7.5
500.00	8.6	9.3

[a] $n = 3$.
[b] $n = 5$.

3.3. Quantification of PFOS and other PPARα ligands with PPRE modified-gold nanoparticle

The polymerization process of silver particles was accelerated as more PPRE modified-gold nanoparticle probes were captured. The optical density produced by gold nanoparticle-based silver enhancement could be observed in a dose-dependent manner with PFOS concentration (Fig. 3a). The absorbance was linearly related with the PFOS concentration between 100 pM and 1 μM ($y = 0.2997x - 0.1531$, $R^2 = 0.9582$), and the detection limit was 10 pM at the signal to noise ration of 3 (Fig. 3b).

To validate the applicability of the PPRE modified-gold nanoparticle probes method for detection of PPARα ligands, PFOA and MEHP were determined by the present bioassay. A good linearity ($y = 0.4619x - 0.0751$, $R^2 = 0.9692$ for PFOA and $y = 0.4658x - 0.1039$, $R^2 = 0.9565$ for MEHP, respectively) at a concentration range from 100 pM to 1 μM was obtained (Fig. 4).

To evaluate the precision of this method, the same PFOS sample was measured consecutively by triplicate experiments to assess intra-day assay precision, and by five consecutive experiments to assess inter-day assay precision (Table 2). The coefficient of variations ranged from 4.8% to 8.6% and from 5.5% to 9.3%, respectively.

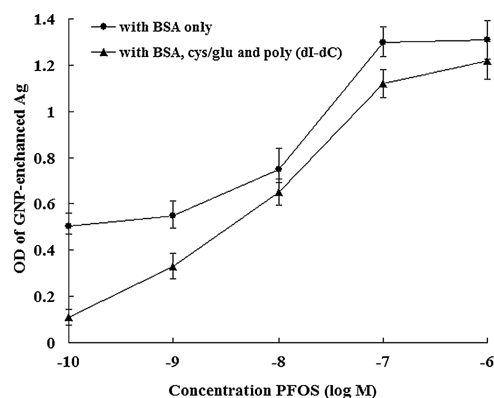

Fig. 2. Reduction of nonspecific binding between gold nanoparticle probes and the receptor protein in the microwells by BSA, cys/glu and poly (dI-dC) optimization: the solid circles (●) represent the optical density value of silver enhancement with the addition of BSA only; the triangle (▲) represents the value with BSA, cys/glu and poly (dI-dC). Each value represents the mean ± SD of triplicate independent determinations. DMSO was used as a control.

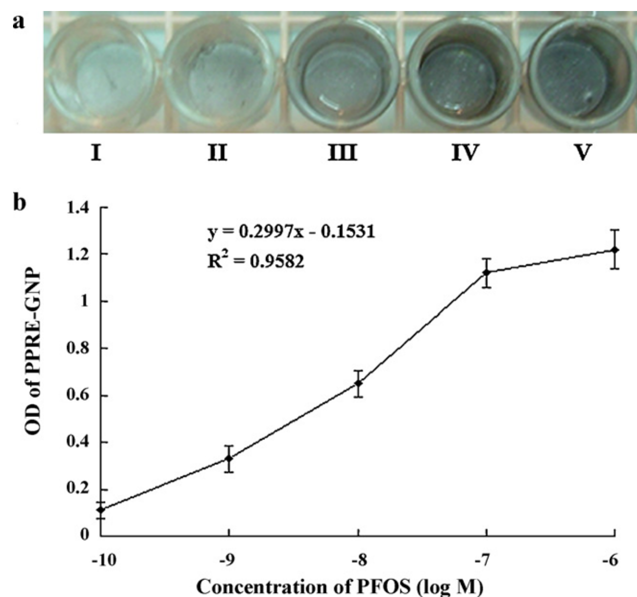

Fig. 3. Optical density of Ag enhancement to serial PFOS concentration with PPRE modified gold nanoparticle probes bioassay: (a) the picture of the color differentiation among different dose groups (I. 100 pM, II. 1 nM, III. 10 nM, IV. 100 nM, V. 1 μM); (b) the linearity between the corresponding optical density (OD) of Ag enhancement and concentration series of PFOS. Each value represents the mean ± SD of triplicate independent determinations. DMSO was used as a control. (PPRE–GNP represents the bioassay of PPRE-modified gold nanoparticle probes with silver enhancement, which is the same in the following figures.)

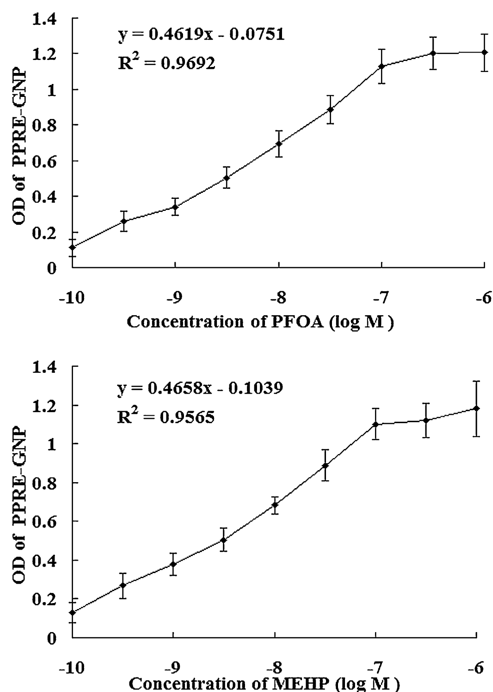

Fig. 4. Detection of other PPARα agonists with PPRE modified nanoparticle probes bioassay. Each value represents the mean ± SD of triplicate independent determinations. 0.05% DMSO was used as a control.

3.4. Comparison of this bioassay with LC–MS for standard and river water samples

In the experiment, the linear range of LC–MS to detect PFOS was 0.02–2.0 μM, and PFOA 0.1–2.0 μM, with the detection limit 0.5 nM and 1 nM from the signal to noise ration of 3, respectively, which were accordant with the previous report [13]. A serial of different concentrations of PFOS spiked ultra-pure water samples were detected by this bioassay and LC–MS, respectively. The results showed quantification data of PFOS samples with good correlation ($r = 0.9893$) between the PPRE modified-gold nanoparticle probes bioassay and the LC–MS method (Fig. 5), and there were no significant difference between them ($P > 0.05$).

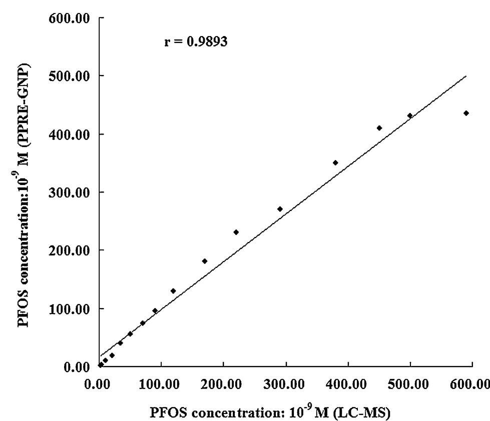

Fig. 5. Concordance of PPRE modified-nanoparticle probes bioassay (PPRE–GNP) and LC–MS quantification of PFOS spiked in ultra-pure water. Each value represents the mean ± SD of triplicate independent determinations.

Through solid phase extraction, the recoveries were calculated using river water fortified with PFOS or PFOA at a concentration of 4 nM ($n = 5$). For the bioassay and LC–MS, the recoveries PFOS were 103.4% [coefficient of variation (CV), 10.5%] and 93.3% (CV, 4.8%), and the recoveries for PFOA were 98.0% (CV, 9.9%) and 92.3% (CV, 4.3%), respectively.

To further verify the efficiency of the PPRE modified-gold nanoparticle probes bioassay in detection of environmental samples, the water samples from Yangtze River were detected by the bioassay and the classic chemical method (LC–MS) (Table 3). The concentration of PFOS and PFOA in river water was detected by LC–MS, respectively. The toxic equivalents (TEQ) [15] of PFOS were used as a marker here to measure the concentration of PPARα ligands in Yangtze River water samples (Table 3). The TEQ of PFOS was calculated by applying a linear regression equation for PFOS determined in the bioassay.

4. Discussion

In this experiment, the liver of male Sprague Dawley rats was chosen to extract the nuclear receptor PPARα, since PPARα was highly expressed in liver cells [28]. From the Western blotting results of nuclear protein extraction solution, the PPARα protein density increased with the concentration of nuclear protein extract product increase. Thus, the nuclear receptor PPARα is expressed highly in hepatocytes.

In order to elevate the specificity of the bioassay, apart from 1% BSA [32], 100 nM cys/glu and 1 μM poly (dI-dC) were used to reduce nonspecific binding. Gold nanoparticles can bind pro-

Table 3
Determination of PPARα ligands in Yangtze River water samples by bioassay and PFOS and PFOA by LC–MS.

Samples	Bioassay	LC/MS	
	TEQ of PFOS (pM)[a]	PFOS (pM)	PFOA (pM)
1	208.2 ± 12.0	36.5 ± 0.8	115.7 ± 2.0
2	225.7 ± 15.2	40.1 ± 0.8	153.1 ± 2.2
3	192.4 ± 13.6	27.3 ± 0.6	109.6 ± 2.5

[a] This concentration of PPARα ligands was considered as the concentration of PFOS which induced an equivalent effect of PPARα activation.

teins nonspecifically, mainly due to the fact that gold nanoparticles can interact with the $-NH_2$ and –SH contained in amino acids of proteins [34]. In order to reduce nonspecific binding between gold nanoparticles and proteins, the cys/glu solution rich in $-NH_2$ and –SH was used to block the remaining binding sites on the surface of the gold nanoparticles which were not covered by BSA. That would produce the most substantial interaction between PPRE and the transcription factor PPARα. On the other hand, poly (dI-dC) with the ability to compete with the labeled probe for binding proteins, was added before the reaction to minimize nonspecific protein–probe interactions, especially when proteins were obtained from nuclear extracts [16,35]. The probes-modified gold nanoparticles were very stable according to previously report [36,37]. In this research, after the probes-modified gold nanoparticles were pre-incubated with 1% BSA, then added cys/glu and poly (dI-dC), there was no aggregation of gold nanoparticles observed.

Moreover, several other important factors should be mentioned in quantification of PPARα ligands to improve the precision. Firstly, the optimal silver-deposition time, data from more than three experiments indicated that silver enhancement terminated at 180 s is suitable. Secondly, it is important to confirm that the captured probes could reflect the ligand–receptor activity correctly. In the experiment, when mutant PPRE-modified nanoparticle probes were added into the reaction system, they were not able to produce effective silver enhancing compared with the specific PPRE probes. Besides, when 1000 times excess cold probes (unmodified PPRE probes) were used to compete with PPRE modified nanoparticle probes (signal probes), the optical density value from silver enhancement produced by captured PPRE modified-gold nanoparticle probes decreased about 95% compared with that from the fully modified PPRE probes. Finally, the nuclear extract and the PPRE-probes can be prepared and stored until use, so that the performance of bioassay will be more convenient.

Based on the technique of silver enhancement of gold nanoparticles probes, the signal was extraordinary amplified because of the polymerizing properties of gold nanoparticles to Ag particles [15,23,26], thus the sensitivity was increased. For detection of standard PFOS and PFOA, the detection limit of the bioassay was 10 pM, which was comparable with the main chemical analysis like LC–MS, LC–MS–MS and GC–MS [10–14]. And there were no significant differences between the bioassay and LC–MS for PFOS spiked ultrapure water. Besides, depending on the acceptable detection linear range for PFOS, PFOA and MEHP, the bioassay would be applicable for monitoring PPARα ligands samples, especially when the concentration was low. Since the three substances of PFOS, PFOA and MEHP have a little different binding affinity to PPARα receptor [38,39], so the response curves for PFOS, PFOA and MEHP are different.

Bioassay is a useful tool in screening environmental samples. Many bioassays have been explored for detecting various chemical pollutants. For example, several ones have been developed for detecting dioxins during the past decades, such as ethoxyresorufin-O-deethylase (EROD) [33], chemical-activated luciferase gene expression (CALUX) [40], and exonuclease protection mediated PCR bioassay (EPM-PCR) [41]. These bioassays are based on receptor–ligand interaction, which cannot distinguish between different target ligands in complex samples, but they can provide biologic potency information of either individual congeners or complex mixtures. Unlike the classical chemical analysis (e.g., LC–MS), the PPRE modified-gold nanoparticle probe bioassay based on the specific binding of PPARα with PPARα ligands would integrate potential activation of other active congeners in a mixture of PPARα ligands. In bioassay, the selection of substance to represent the TEQ is very important. Although PFOS is not the strongest PPARα ligand, it is one kind of the most common and hot research environmental pollutants, the toxicity of which through PPARα has been studied widely. Here, in order to better compare the performance of the bioassay for environmental water samples with LC–MS, the concentration of PFOS was selected as TEQ to evaluate. The concentration of PFOS and PFOA in river water were selected to detect by LC–MS, since the two compounds were the most dominating PPARα ligands in environment [42] and reported with high concentration in Yangtze River [43]. The TEQ of PFOS detected by the bioassay was higher than the total concentration of PFOS and PFOA by LC–MS, since there were other PPARα ligands existed in environment.

This PPRE-modified gold nanoparticle probe based bioassay provides a useful platform for the screening of PPARα ligands in environmental matrices. It is useful especially when there are a large number of unknown samples waiting for detection due to the high sensitivity and wide detection range of the bioassay. Positive samples could then be made further analyses with LC–MS to identify the quantity of specific compounds if it is necessary. Utilizing this bioassay to screen out the negative samples, the subsequent expense of detection for PFOS, PFOA, or other members of PPARα ligands, is likely to be cut down largely by avoiding analyzing negative samples with LC–MS. Moreover, this bioassay allows TEQ analysis without a very complicated protocol or expensive instruments. The prerequisite instrument, a microplate reader, is common in many laboratories due to the similarity of the procedure to ELISA.

Besides, it is important to note that there are some other PPARα ligands like lipid regulating drugs (e.g., clofibrate) widely used for carcinoma therapy. Although they are not regarded as persistent pollutant at present, they were detected in sewage sludge and even in drinking water recently, which is posing potential threat for human health [40]. This bioassay presented here can also provide positive results if these chemical with potential hazard presented in samples.

5. Conclusion

This study demonstrates a new protocol of a cell-free bioassay based on silver enhancement of gold nanoparticles and the interaction among ligands, PPARα and PPRE for rapid and sensitive detection of PPARα ligands. The method can be used in conjunction with LC–MS. There are several advantages of this biological method. Firstly, it is performed in 96-well microplate, so it can be used for screening many environmental samples simultaneously. Secondly, as a biological method for measuring and screening PPARα ligands from samples, it will only cost several hours to finish the protocol if the preparation work is sufficient. Thirdly, the method is promising for on-site measurement, to be used for pre-screening and analysis of PPARα ligands in environmental samples.

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