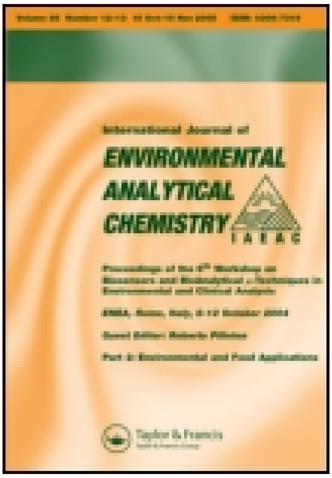

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Oestrogen receptor binding assay is an important approach to screen oestrogenic endocrine disruptors. But it is often expensive and radioactive pollution has existed. In order to screening endocrine disrupting chemicals (EDCs) without a radioactive label, we developed a new high-throughout method using gold nanoparticle technology. The assay is based on the competition binding between the oestrogenic EDCs in the sample and 17β-estradiol-BSA to the oestrogen receptor. The signal is from specific binding of gold nanoparticles labelled ERE to the ligand-receptor complex. The result showed that as little as 100 pg L^{-1} of 17β-estradiol could be detected with a linear range from 100 pg L^{-1} to 1 μg L^{-1} ($R^2 = 0.9764$). The concentrations of oestrogenic EDCs in environmental sample determined by our method and by the cell (MCF-7) proliferation were not significantly different. The result presented led us to conclude that this method is an ideal screening method which is reliable, low-cost, rapid, high-throughout and could be performed on microplates or chips.

Keywords: oestrogenic EDCs; 17β-estradiol; oestrogen receptor; oestrogen receptor element; gold nanoparticle

1. Introduction

A growing body of scientific research indicates that many natural and man-made industrial chemicals may interfere with the normal functioning of human and wildlife endocrine, or hormone, systems. These endocrine disrupting chemicals (EDCs), which persist in the environment due to their structure stability, may cause a variety of problems with development, behaviour and reproduction [1]. A large part of EDCs, including pharmaceuticals, pesticides, industrial chemicals and heavy metals, have been identified that induce oestrogen-like responses and are classified as oestrogenic EDCs [2]. Oestrogenic EDCs could mimic the gonadal hormone 17β-estradiol and interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour. Most oestrogens are retained with high affinity and specificity in target cells by an oestrogen receptor (ER), which functions as a ligand-modulated transcription regulator. In the target tissue, the binding of oestrogen to ER will induce the ER conformation changes, dimerisation and binding to specific sites in the

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promoter region of oestrogen-responsive genes [3], which is an ER element (ERE) [4,5]. Transcription of the target genes is initiated by coactivators and other regulatory proteins that bind to the external surface of the dimer ER complex.

Oestrogen receptor binding assay, which is preferred as Tier 1 Screening method for endocrine disruptors by Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), is an important approach to screen oestrogenic EDCs. It relies on the ligand binding of oestrogenic EDCs to the oestrogen receptor. Competitive binding to oestrogen receptors is one of the most popular endpoints during the initial screening stage, since most oestrogenic EDCs will compete with the endogenous oestrogens for binding to an oestrogen receptor. At present, competitive binding assay mainly based on oestrogen receptors and the radioactively labelled 17β-estradiol are used. These assays enable rapid screening of oestrogenic EDCs, but due to the radioactive label, they are often expensive and radioactive pollution exists [6].

With the recent advances in nanotechnology, gold nanoparticles were favoured in the field of bioanalysis for their various characteristics, including no radioactivity, high electron density and high sensitivity [7–9]. Biomolecules, such as oligonucleotide probes capped with alkanethiol groups at their ends, are able to attach themselves covalently to nanoparticles [10,11]. Niemeyer *et al.* [7] have done much work on DNA-gold nanoparticle-based protein detection. In the present study, we developed a new high-throughout screening method for detection of oestrogenic EDCs that uses gold nanoparticles as a signal label. The assay relies on the competitive binding of oestrogenic EDCs to the oestrogen receptor with 17β-estradiol-BSA. Gold nanoparticles labelled ERE can recognise the ligand and receptor complex. The application of nanoparticle enables sensitive detection of oestrogenic EDCs without a radioactive label.

2. Experimental

2.1 *Materials and oligonucleotide probes*

Oligonucleotides modified with thiol group were purchased from Invitrogen, Inc. (Shang Hai, China) and used without further purification. Gold nanoparticles with diameters of 15 nm, 17β-estradiol-BSA conjugate, 17β-estradiol, recombinant human oestrogen receptor-α, XAD-2 resin were purchased from Sigma (St. Louis, USA). The anti-human ER-α polyclonal antibodies (anti-ER) were obtained from Santa Cruz Biotechnology (California, USA). BSA was obtained from Amersco (Ohio, USA). $AgNO_3$, citric acid, trisodium citrate and hydroquinone were purchased from Sheng Gong (Shang Hai, China). MCF-7 cell was purchased from the China Center for Type Culture Collection (Wu Han, China). (In order to keep the activity of the materials in the reaction, we prepared the materials with a PBS buffer, and stored them at 4°C or −20°C according to the storage requirement of each material.)

2.2 *Preparation of gold nanoparticle-modified ERE probes*

Sequence 1. 5′-SH-$(CH_2)_6$–GTATGTA<u>GGTCA</u>CTG<u>TGACC</u>CCCGA-3′,
Sequence 2. 5′-TTTTTC GGG<u>GGTCA</u>CAG<u>TGACC</u>TACATAC-3′.

The underlined sequences correspond to the oestrogen-ER binding core site and are referred to as ERE sequences [12–14].

Sequence 1 (100 µM, 4 µL) dissolved in 46 µL Tris-Cl/EDTA (TE) buffer (pH 8.0) was added to the precipitated of 500 µL gold nanoparticles (stored at 4°C and kept in dark places), which were centrifuged at 14,300 rpm, 12,000 × *g* for 20 min, and mixed quickly and thoroughly. The mixture was stored at 4°C for 24 h, followed by addition of 50 µL buffer (0.2 M NaCl, 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.0) and stored at 4°C for another 24 h. The solution was centrifuged at 14,300 rpm for 10 min. Unlabelled oligonucleotide probes were put in the supernatant and removed. Then, the gold nanoparticle-modified sequence 1 were all dissolved in 100 µL buffer (0.1 M NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.0) and stored at 4°C. Before the experiment, gold nanoparticle-sequence 1 was hybridised with sequence 2 for 4 h at 25°C in a solution containing 1.5 M NaCl, 50 mM PBS and 0.05% SDS to form double-stranded gold nanoparticle-ERE probe.

2.3 *Surface treatment of the microplates*

17β-estradiol-BSA conjugate or anti-ER was immobilised to the surface of microplates (pre-blocked with 5% BSA). 17β-estradiol-BSA conjugate (20 µg mL^{-1}, working solution 1 : 100) or anti-ER (1 : 100) diluted with 100 µL of carbonate buffer ($NaHCO_3/Na_2CO_3$ 50 mM, pH 9.6) were dispensed into each well of the 96-microplate. The microplates coated with 17β-estradiol-BSA conjugate (M 1) or anti-ER (M 2) were incubated at 4°C overnight and then washed with 300 µL washing solution (0.05% Tween 20 in 10 mM PBS, pH 7.6) three times. The plates were blocked for 1 h with blocking solution (10 mM PBS containing 3% BSA, 0.05% casein and 0.05% Tween 20, pH 7.6) and washed as described before.

2.4 *Quantification of oestrogenic EDCs*

The chemical solutions containing 17β-estradiol (it is used for making calibration curve) of known concentrations (dissolved in methanol and diluted with PBS, 100 µL per well) or sample including oestrogenic EDCs were added together with the ER (1 : 2000) into M 1 and incubated at 20°C for 1 h, for competitive ligand-receptor binding reaction. After this, the supernatant, which contains ER-17β-estradiol complex or ER-oestrogenic EDCs complex, was added into M 2 and incubated at 20°C for 1 h to complete the antigen-antibody binding. Then the plates were rinsed with washing solution three times. The gold nanoparticle-ERE diluted at 1 : 20 were added to microplate and incubated at 20°C for 1 h and then rinsed with washing solution for 3 times and PBN (0.3 M $NaNO_3$ and 10mM PBS, pH 7.6) for 3 times. Signal enhancement was carried out by incubating the microplates with 100 µL enhancer solution [7] (0.5 g $AgNO_3$ in 2 mL H_2O, 1.7 g hydroquinone in 30 mL H_2O, 2.55 g citric acid, and 2.35 g trisodium citrate in 10 mL H_2O, all mixed together simultaneously immediately before use) at 25°C. The enhancement time was 60–160 s. The reaction was ended by immersing the microplates in doubly distilled water [15].

2.5 *Comparison of gold nanoparticle-modified ERE assay with MCF-7 cell proliferation assay*

The pretreatment of real water sample was performed as follows: each lake water sample (120 L) was passed through chromatographic column at a flow rate of 20 mL min^{-1} under positive pressure. The columns (2 cm diameter) were filled with XAD-2 resin. Each sample was dried under a gentle nitrogen stream for 1 h and eluted with methanol (60 mL) and *n*-hexane (60 mL). Extracts were collected into a glass flask, evaporated in Rotary Evaporators, and resuspended in 2 mL methanol. The samples were detected qualitatively by gold modified-ERE assay and MCF-7 cell proliferation assay. MCF-7 cell proliferation assay were performed as described by Soto [16].

3. Results

3.1 *Design strategy of gold modified-ERE assay*

Gold modified-ERE assay is based on the competition between the oestrogenic EDCs in the sample and natural oestrogen (17β-estradiol-BSA) which is immobilised on the microplate binding to the ER. As shown in Figure 1(a), the sample is incubated together with a limited amount of ER. After the receptor competitive binding reaction, the ER-oestrogen complex is transferred and captured by anti-ER antibody, which is immobilised on another microplate. Gold nanoparticle-modified ERE probes are subsequently bound to the ER-oestrogen complex. The signals, whose intensity is proportional to the quantity of oestrogenic EDCs in the sample, are amplified by silver enhancement and detected with optical density. After the amplification, the result could be observed visually (Figure 1(b)).

3.2 *Characteristics of the gold nanoparticles*

Since gold nanoparticles could self-assemble into branched nanostructuresin by the light irradiation [17], gold nanoparticles were characterised by transmission electron microscopy before preparation of gold nanoparticle-modified ERE probes. As shown in Figure 2, the gold nanoparticles possessed high dispersion stability and an average diameter of 15 nm.

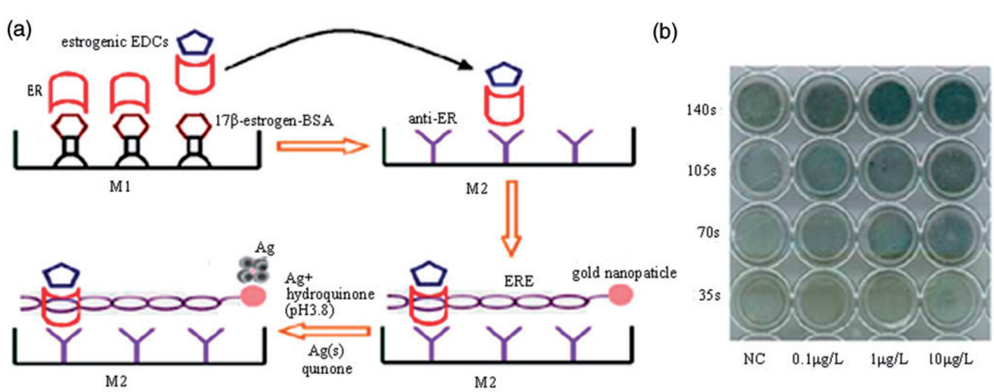

Figure 1. (a) Design strategy of GNEREA, (b) microplate image illustrating the detection of different concentrations of oestrogenic EDCs.

3.3 *Set up calibration curve by 17β-estradiol with gold modified-ERE assay*

Serial dilutions of the 17β-estradiol were used as standard to construct the calibration curve. As shown in Figure 3, the signal (absorbance at 490 nm) was linear related to 17β-estradiol concentration from 100 pg L^{-1} to 1 μg L^{-1} with a detection limit of 100 pg L^{-1}. The linear regression equation was $Y = 0.1977x + 0.2767$, where Y was

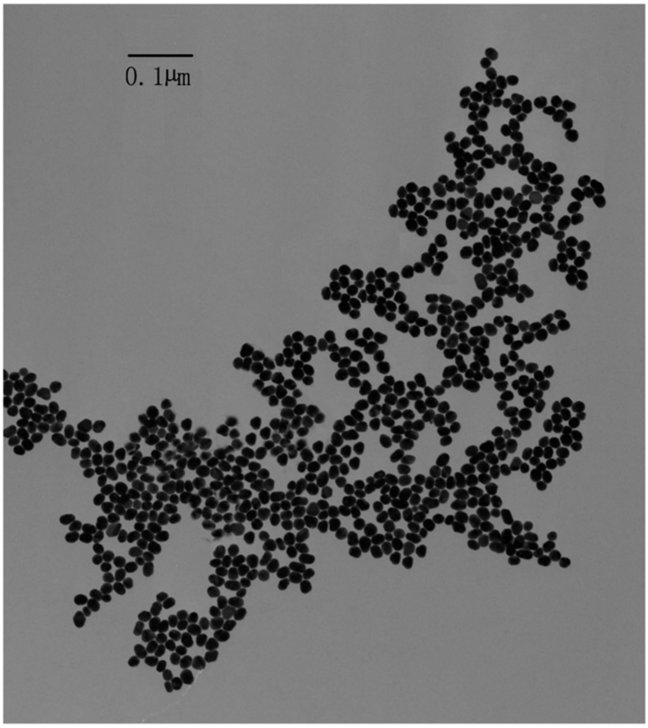

Figure 2. TEM image of 15 nm gold nanoparticles.

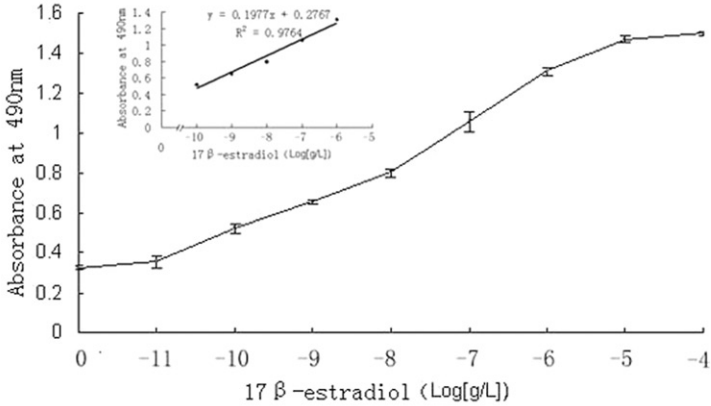

Figure 3. Calibration curve of a dilution series of 17β-estradiol with gold modified-ERE assay.

Table 1. Intraassay and interassay imprecision for gold modified-ERE assay.

	Coefficient of variation, %[a]	
Estrogen concentration	Intrassay	Interassay
100 pg L^{-1}	4.78	2.45
1 ng L^{-1}	1.98	1.56
10 ng L^{-1}	2.55	3.62
100 ng L^{-1}	4.54	3.15

Note: [a]$n = 5$.

the absorbance, x was the logarithm of the 17β-estradiol concentration. The correlation coefficient (r^2) was 0.9764.

3.4 *Assay precision*

We evaluated the method's intraassay imprecision by consecutively analysing the same concentration of the 17β-estradiol in five replicates and assessed interassay imprecision by analysing the sample on five consecutive days. The results are shown in Table 1. The intraassay imprecision (CVs) was $\leq$4.78% and interassay imprecision (CVs) $\leq$3.62, demonstrating an acceptable level of precision.

3.5 *Comparison of gold modified-ERE assay with MCF-7 cell proliferation*

The estradiol equivalency factor (EEF: its scheme weighs oestrogen effect of the compounds as fractions of the oestrogen effect of the 17β-estradiol. This factor indicates the degree of oestrogen effect compared to 17β-estradiol, which is given a reference value of 1. To calculate the total oestrogenic EDCs of a mixture, the amounts of each oestrogenic EDC are multiplied with their EEF and then added together) of oestrogenic EDCs in the water of East Lake were detected by gold modified-ERE assay and MCF-7 cell proliferation to compare the two methods. The results obtained from gold modified-ERE assay significantly correlated with those obtained from MCF-7 cell proliferation ($r^2 = 0.9657$) (Figure 4).

4. Discussion

Many man-made chemicals with oestrogenic activity are used extensively in major industries, including agriculture, the petrochemical industry, the plastic industry, the pharmaceutical industry, the detergent industry, and so on. Thus, oestrogenic EDCs have become a major issue in recent years.

Current detection methods for oestrogenic EDCs include chemical analytical methods and biologically based assays. The chemical analytical methods, such as HPLC, GC/MS and LC-MS/MS [18–20], provide excellent sensitivity and precision for monitoring environmental oestrogen. However, these techniques measure specific oestrogenic EDCs individually, so the target compound must have already been identified as have oestrogenic

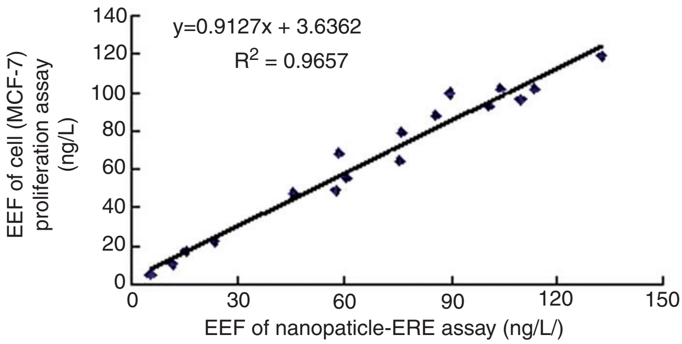

Figure 4. Comparison of gold modified-ERE assay and cell (MCF-7) proliferation for detection of oestrogen in samples.

properties [21]. Biologically based assays, such as cell proliferation, ligand binding [16], vitellogenin induction [22], luciferase induction [23] and antigen-antibody interaction [22], provide alternative detection methods to traditional analyses. But there is always lack of specificity of organism response to various oestrogenic EDCs [21]. Cellular assays are confronted with the problem of cytotoxic substances, which may be present in environmental samples and could lead to erroneous results, unless proper controls are included [6].

Based on oestrogenic receptor binding assay, we developed a novel densitometric assay for detection of oestrogenic EDCs. It is based on the competitive binding of oestrogenic EDCs to oestrogen receptor with 17β-estradiol. Immobilised 17β-estradiol-BSA was used as the ligand in the assay. In this assay, the gold nanoparticle modified ERE are specifically bound to ligand-ER dimeride complex. When transferred into the anti-ER antibody coated microplates, the antigen-antibody ligand-ER-ERE complex can be immobilised onto the surface of microplates via antigen-antibody interaction. The excess ERE-gold nanoparticles can be washed out to ensure the accuracy of later quantifications. Because the assay could be performed on microplates or chips and the gold nanoparticles, it enables screening of oestrogenic EDCs in a high-throughout manner without radioactive contamination. The gold nanoparticles serve as a powerful signal label for nucleic acids and proteins detection, as gold nanoparticles have high catalysis activity and good biocompatibility. In this assay, gold nanoparticles catalysed the reduction of silver ions to metallic silver on the particle surface. The darkening of gold nanoparticles by the silver enhancement largely improved the sensitivity of the assay, which was approximately $2 \sim 3$ times higher than the oestrogen receptor binding assay that existed [24].

The human ER-α, stored at $-70°C$, was used as binding receptor in this method. The optimised reaction temperature was set at 20°C, as the recombinant human ER would be deactivated when the temperature exceeded 37°C. Another point should be noted is the quantity of 17β-estradiol-BSA conjugate should be excess over the quantity of ER. Otherwise the excessive ER which does not bind to 17β-estradiol-BSA will bind to the gold nanoparticle-modified ERE and interfere with the result.

Because the concentrations of oestrogenic EDCs in environmental samples are always very low, the signals of gold nanoparticles need to be amplified to provide better analytical performances. In this method, to facilitate visualisation of the gold nanoparticles fixed to the surface of the microplates via ER and ER antibody interaction, we used silver-enhancement technology to amplify the signals of gold nanoparticles and it enables the oestrogenic EDCs detection down to picogram range. Temperature is an important factor in silver enhancement. We optimised conditions for silver enhancement as reacting for 100–140 s at 25°C under a red light, which reduced self-nucleation of silver ions greatly. It also should be noted that the microwells must be washed thoroughly with PBN prior to silver enhancement to remove chloride ions, which could bind to silver ions to form AgCl and interfere with the silver enhancement process.

5. Conclusions

In conclusion, our method provides a rapid, quantitative and low-cost method for oestrogenic EDCs detection. The method is performed in 96-well microplate, so it could be used for high-throughout detection of oestrogenic EDCs. With the gold nanoparticles label and Ag enhancement technology, the sensitivity is improved significantly, so it may serve as a sensitive method for oestrogenic EDCs detection. In addition, the method may also contribute to the growing number of applications of nanotechnology.

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