Time-resolve Fluorescence Immunoassay for Determination of Diethylstilbestrol Based on Dual-codified Gold Nanoparticles

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Abstract. An ultrasensitive time-resolve fluorescence immunoassay for the determination of diethylstilbestrol (DES) was established based on dual-codified gold nanoparticles (DC-AuNPs). The dual-codified gold nanoparticles was composed of gold nanoparticles (AuNPs) conjugated to anti-DES antibody and SH-dsDNA-biotin. The DES-OVA immobilized on polystyrene microtitration plates and the DES in the standard solution or sample were competitively reacted with the anti-DES antibodies immobilized on DC-AuNPs, and the fluorescence signal was amplified via AuNPs and biotin-streptavidin (B-SA) dual amplification system; followed by the addition of enhancement solution, the Eu³⁺ was disengaged from the reactive product and formed new chelate with the enhancement solution again, and the fluorescence signal was further enhanced. Under the optimized condition, the method shows a wide linear range from 1.0×10⁻⁶ to 10.0 ng/mL with detection limit of 0.4 fg/mL. This method was applied to determine DES in milk sample, with the percent recoveries in the range of 91.9-101.5%.

Introduction

Diethylstilbestrol (DES), a synthetic estrogen hormone, was firstly synthesized by Dolds in London, and found to be the most potent endocrine disruption chemicals (EDCs). From 1940 to 1971, DES was used in USA as a treatment to prevent miscarriages and that often is used for clinical therapy. Later, it was widely used as a growth promoter in cattle and calves¹, because DES can increase the weight gain of animals. In addition, it was also used as a treatment for estrogen-deficiency disorders in veterinary medicine, more recently, for postcoital contraception. After it is used, a portion was discharged into the water environment by excretion and the rest remains in the animal’s body, so it may be transferred into water, foods, and food webs if it was not well controlled. And the mass of evidence proved that it may lead to potential risk to humans and wildlife, even at low levels. Therefore, from 1976, many countries had banned the use of DES due to its teratogenic, mutagenic, and carcinogenic properties². Recently, menarche age of teenagers is earlier obviously compared with ten years ago, and the quality of human sperm are declined obviously, which are associated with eating the food contain DES for a long time. Consequently, it is great important to detect trace levels of DES and their metabolites to control their abuse, which entails analytical methodology capable of high sensitivity.

Up to now, various analytical methods for the detection of DES have been developed, such as high performance liquid chromatography (HPLC)³, HPLC-fluorescence detection (HPLC-FLD)⁴, flow injection chemiluminescence method (FIC)⁵, electrochemical analysis (ECL)⁶. And various immunochemical methods also have been established for the determination of DES, including radioimmunoassay (RIA)⁷, enzyme-linked immunoassay (ELISA)⁸, time resolved fluoroimmunoassay (TRFIA)⁹, etc. However, The GC-MS and HPLC-MS are sensitive and
specific, they are expensive and not accessible in ordinary laboratories; RIA presented a certain risk and ELISA can be used only as a screening method since it tends to overestimate the concentration. TRFIA can offer better sensitivity than ELISA and other traditional analytical methods, due to the unique fluorescence properties of the lanthanide chelates and the time-resolved measurement mode that enable the specific fluorescence to be measured after the background fluorescence has already declined. Because of the high sensitivity, rapidity of reaction, simple instrumentation, and wide dynamic range, TRFIA has been used as an attractive analytical method in different fields, such as biotechnology, pharmacology, molecular biology, and clinical environmental chemistries. Nevertheless, the sensitivity of TRFIA needs to be further improved since the residue level of DES in food and environment is usually very low.

Gold nanoparticles (AuNPs) have been explored for potential applications in biomolecular detection owing to huge reactive surface area, unusual catalytic property, localized surface plasmon resonance, fluorescence quenching ability, and biocompatible\[^{[10]}\] and amenability to different sensing methods\[^{[11]}\]. With the rapid development of AuNPs functionalization\[^{[12]}\], AuNPs has been the most widely used for immunoassay as carriers, and greatly amplify fluorescence signal.

In this study, a novel, rapid, and ultrasensitive dual-codified gold nanoparticles consisting of gold nanoparticles conjugated to both thiol-DNA and anti-DES antibodies (DC-AuNPs) was developed and validated for the determination of DES with a competing mode by using TRFIA method. As a consequence, the sensitivity of the proposed method was improved, and this method has already been affirmed in the analysis of the milk samples.

**Experimental**

**Apparatus**

In this thesis, the apparatus used include the Infinite 200 auto multifunction microplate reader (TECAN, Switzerland), polystyrene microtitration strips with 96 flat-bottomed wells (Costar), WDT model rocking incubator with thermostatic controller, pHS-3C digital pH meter (Dazhong Analytical Instruments Co., Shanghai, China), XW-80A Commix (Luxi Analytical Instruments Co., Shanghai, China), 78-1 Magnetic stirrer (Jiantan Medical Instruments Co., Jiangsu, China), TGL-16B Centrifuge (Anting Analytical Instruments Co., Ltd., Shanghai, China), Himac CR 22G High-speed refrigerated centrifuge (Hitachi Co., Japan), UV-2550 UV-vis spectrometer (Shimadzu Instruments Co., Kyoto, Japan), and CM-120 Transmission electron microscopy (TEM) (Philips, Netherlands).

**Reagents**

All DNA, used in the present work were synthesized and purified by Shanghai Sangon Biotechnology Co., Ltd. Tetrachloroauric acid trihydrate (HAuCl\(_4\)-3H\(_2\)O) and tris (hydroxymethyl)-aminomethane (Tris) were supplied by J & K China Chemical Co., Ltd. Polyclonal rabbit antibody against DES with no significant cross reactivities with other chemicals was obtained from our laboratory. The antigen (DES-OVA) was prepared by our lab. DES, ovalbumin (OVA), diethylenetriaminopentaacetia acia (DTPAA), trioctyphosphine oxide (TOPO), 4,4,4-tri-fluoro-1-(2-naphthyl)-1,3-butanedione (2-NTA), europium(III) chloride (EuCl\(_3\)), Triton X-100, streptavidin (SA) and Tween-20 were bought from Sigma Chemicals Co. (St. Louis, Mo, USA). Sodium citrate was obtained from Beijing Chemical Works (Beijing, China).

All other chemicals were of analytical reagent grade and deionized water (≥18 MΩ·cm) was prepared using a Millipore water system.

**Preparation of Europium(III)-labeled Streptavidin (Eu\(^{3+}\)-SA)**

The streptavidin were labeled with europium(III) chelate in two steps.

The first step: 1.0 mg streptavidin was dissolved in 400.0 μL NaHCO\(_3\) solution (0.20 mol/L, pH 8.5), 50.0 μL DTPAA solution (20 mg/mL) was added to the above streptavidin solution. The mixed solution was incubated for 8 h at 4 °C and then for 2 h at room temperature with gentle stirring,
lastly, the solution was dialyzed for 12 h with normal saline solution (pH 7.4), and the reaction product was SA-DTPAA solution.

The second step: 70.0 μL EuCl₃ (33.0 mmol/L) was added into the above solution. The mixture was incubated 3 h at room temperature with gentle stirring. Then the solution was dialyzed four days by TBS solution (0.05 mol/L, pH 7.8). The final product Eu³⁺-SA was stored at –20 ºC until used.

**Preparation of Dual-codified Gold Nanoparticles (DC-AuNPs)**

AuNPs modified with DNA and anti-DES antibodies via electrostatic interaction and the Au-S bond, respectively. First of all, AuNPs solution was adjusted to the optimal pH by directly using 0.1 mol/L K₂CO₃ solution; Anti-DES antibodies (1.0 mg/mL) and thiol-modified single-stranded DNA (100.0 μmol/L, SH-ssDNA) were added to 1.0 mL gold nanoparticles solution at the same time, gently shake for 30 min before incubating 8 h at room temperature. After the addition of NaCl (2.0 mol/L, dissolved in 0.01 mol/L PB, pH 7.0) to a final concentration of 0.05 mol/L NaCl, the mixture was then incubated overnight at 4 ºC. To remove the unbound anti-DES antibody and SH-ssDNA, the mixture was washed and centrifuged at 14,000 rpm for 20 min twice, and the supernatants were removed. Then, the precipitates were resuspended in hybridization buffer containing 10.0 mmol/L Tris, 1.0 mmol/L EDTA, 0.05mol/L NaCl and 0.05% PEG (w/v), pH 7.4. Afterward, the biotin-modified single-stranded DNA (the complement of SH-ssDNA, B-ssDNA) was injected, followed by a 18 h incubation period at room temperature to hybridized with the ssDNA of the AuNPs coordinated to the NPs and purified using a similar centrifugation procedure. Finally, the product, DC-AuNPs, was resuspended in assay buffer and stored at 4 ºC for further use.

**Competitive Time-resolve Fluorescence Immunoassay**

Polystyrene microtitration plates were coated with 100.0 μL DES-OVA in coating buffer at 4 ºC overnight. Before used, the plates were washed three times with 0.05 mol/L tris washing buffer (pH 7.8) and blocked with 150.0 μL OVA at 37 ºC for 40 min. The plates were then washed as described above and 50.0 μL serial dilutions of the DES (or the solution of sample) and 50.0 μL the complexes of DC-AuNPs were added to the coated wells. After incubation on a shaker at 37 ºC for 1 h with gentle shaking and washing, 100.0 μL 1:500 Eu³⁺-SA was added into each wells, followed by incubation 40 min at 37 ºC. The plates were then washed six times to remove the unbound reagent. Afterwards, 200.0 μL enhancement solution was added. The plates underwent shaking for 5 min and then laid still for 15 min. Finally, the fluorescent intensity was measured under the excitation of 340 nm, the emission of 615 nm and the lag time 200 μs.

**Results and Discussion**

In this study, DC-AuNPs was composed of AuNPs conjugated to anti-DES antibody and SH-dsDNA-biotin via the electrostatic adsorption and the covalent bonding, respectively. The DES-OVA immobilized on polystyrene microtitration plates and the DES in the standard solution or sample were competitively reacted with the anti-DES antibodies immobilized on DC-AuNPs; and the fluorescence signal was stepped amplified via the AuNPs and the biotin-streptavidin (B-SA) dual amplification system; followed by the addition of enhancement solution, the Eu³⁺ was disengaged from the reactive product and formed new chelate with the enhancement solution again, and the fluorescence signal was further enhanced and measured.

**Optimization of Assay Condition**

The Concentration of Coating Antigen. The concentration of coating antigen (DES-OVA) was optimized by gradient method. The coating antigen was diluted by 0.05 mol/L sodium bicarbonate buffer (pH 9.6) into 10.0, 20.0, 40.0, 60.0, 100.0 μg/mL. Different concentration of the coating antigen was coated in the microtitration wells. Followed the immunoassay progress, fluorescence intensity increased with coating antigen concentration increased to 40.0 μg/mL. After that, decreased fluorescence intensity were observed with increasing coating antigen concentration.
When the concentrations of coating antigen was 40.0 μg/mL, fluorescence intensity reached the highest value. Therefore, the concentration of coating antigen was fitted to 40.0 μg/mL.

**The Concentration of Dual-codified Gold Nanoparticle (DC-AuNPs).** The concentration of DC-AuNPs is another important factor to the sensitivity and working range. Thus, eight concentrated ratio (the volume ratio of Au/DC-AuNPs) from 1:1 to 8:1 was tested. The fluorescence intensity increased with concentrated ratio of DC-AuNPs increased. When concentrated ratio of DC-AuNPs was 4:1, the fluorescence intensity reached the strongest. The results indicated that the optimal concentrated ratio was 4:1.

**Effect of Enhancement Solution.** Enhancement solution has a direct effect on the sensitivity of the immunoassay. Because, the Eu ion can disengage from the reactive product by the enhancement solution and form new chelate with the enhancement solution again. The quantity of the enhancement solution, which was added in the microtitration wells, can influence the extent of the disengaging of the Eu ion and the fluorescence intensity of the new chelates. Followed the immunoassay progress, the fluorescence intensity of the new chelates was investigated. As shown in the test, with the volume increasing from 100.0 to 280.0μL, the signal was gradually enhanced and reached a peak, then showed peak attenuation. The optimum volume of the enhancement solution was therefore determined as 200.0 μL.

**Standard Curve**
Under optimal conditions, a standard curve was completed with the DC-AuNPs-TRFIA method. The curve showed a good response for DES at concentrations of 1.0×10⁻⁶-10.0 ng/mL. The linear regression equation is ΔI(I₀-I)=689.0lgC⁻⁰⁹⁺²⁺+6221.9, the linear correlation coefficient was R²=0.9967, and the detection limit (LOD) was 0.40 fg/mL.

**Analysis of Samples**
Diethylstilbestrol was may added into the animal feed as a growth promoter by illegal businessmen, which may resulted that residual diethylstilbestrol existed in milk as excretion of dairy cow and maintain a direct biological activity. Therefore, the detection of diethylstilbestrol in milk is necessary. Recoveries of milk spiked at 0, 0.1, 1.0, 2.0 and 5.0 ng/mL were examined with three replicates using diluent as control. The results demonstrated that the recoveries for DES in spiked samples were over the range of 91.9-101.5%. The recoveries and relative errors have confirmed that it has fine accuracy and broad utility of this proposed method in real samples.

**Conclusions**
In summary, a new competitive DC-AuNPs-TRFIA method was proposed as a quantitative and rapid immunoassay for the detection of DES. This method was firstly applied to the detection of DES, and the result correlated well with the FIA. In addition, this method does well in linearity, sensitivity, precision, recovery, specificity and viability, which were in accord with the requirement of immunoassay. Therefore, it can be commendably used in the detection of DES. The complexes of DC-AuNPs and europium(III)-labeled streptavidin (Eu³⁺-SA) was regarded as a probe in this system. Optical sensitivity enhancement was attributable to the use of gold nanoparticles as a multi-dsDNA-label carrier and the streptavidin-biotin system, which therefore amplify the fluorescence signal, represents the most important achievements due to the use of this dual-codified nanolabel, which can potentially be exploited in several other future applications.

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References


