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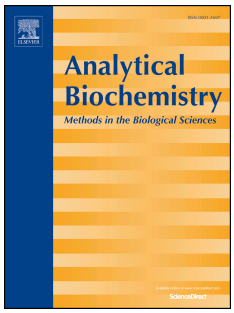

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Colorimetric aptasensor for progesterone detection based on surfactant-induced aggregation of gold nanoparticles

Gaoshang Du[a] • Lumei Wang[a]* • Dongwei Zhang[a] • Xuan Ni[a] • Xiaotong Zhou[a] • Hanyi Xu[a] • Lurong Xu[a] • Shijian Wu[b]• Tong Zhang[a] • Wenhao Wang[a]

Abstract

This paper proposes an aptasensor for progesterone (P4) detection in human serum and urine based on the aggregating behavior of gold nanoparticles (AuNPs) controlled by the interactions among P4-binding aptamer, target P4 and cationic surfactant hexadecyltrimethylammonium bromide (CTAB). The aptamer can form an aptamer-P4 complex with P4, leaving CTAB free to aggregate AuNPs in this aptasensor. Thus, the sensing solution will turn from red (520 nm) to blue (650 nm) in the presence of P4 because P4 aptamers are used up firstly owing to the formation of an aptamer-P4 complex, leaving CTAB free to aggregate AuNPs. However, in the absence of P4, CTAB combines with aptamers so that AuNPs still remain dispersed. Therefore, this assay makes it possible to detect P4 not only by absorbance measurement but also through naked eyes. By monitoring the variation of absorbance and color, a CTAB-induced colorimetric assay for P4 detection was established with a detection limit of 0.89 nM. Besides, the absorbance ratio *A*650/*A*520 has a

[a]School of Agriculture and Biology, and Key Laboratory of Urban Agriculture (South), Ministry of Agriculture, and Bor S. Luh Food Safety Research Center, Shanghai Jiao Tong University, Shanghai, 200240, PR China.

[b]Shanghai Environmental Monitoring Center, Shanghai 200235, PR China.

[*]Corresponding author, E-mail: zjuwlm@163.com; Tel: +86-21-34206924; Fax: +86-21-34205762.

linear correlation with the P4 concentration of 0.89–500 nM. Due to the excellent recoveries in serum and urine, this biosensor has great potential with respect to the visual and instrumental detection of P4 in biological fluids.

Keywords: gonadal hormone, aptamer, biosensor, AuNPs, rapid testing, visual test

1. Introduction

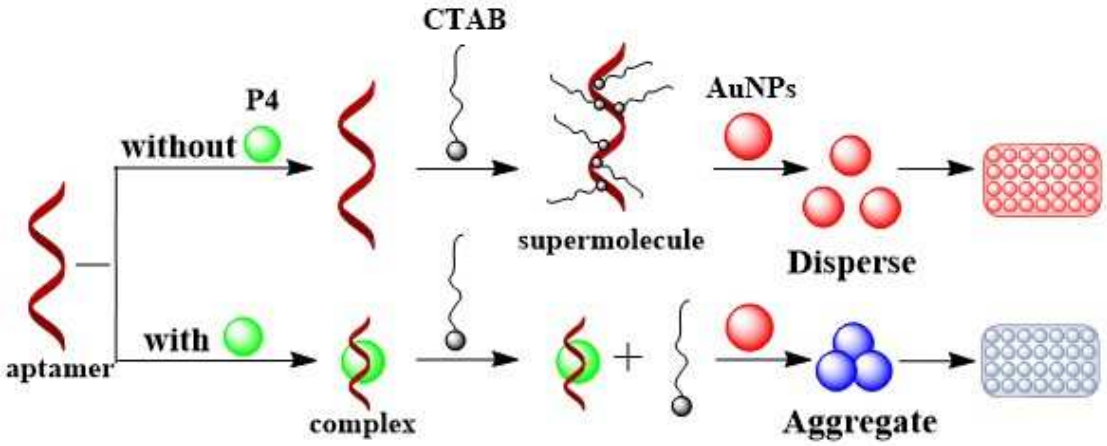
Progesterone (P4) is a steroid hormone that is essential for the development of mammary tissue and maintenance of pregnancy. It also helps female regulate the menstrual cycle, and plays an important role in menopausal hormone therapy and oral contraceptives [1,2]. P4 is an indispensable for human body and the concentration varies in different period of life. For example, the concentrations of serum progesterone in adult females are normally in the range of ~0.48 to ~79.5 nM, while the P4 concentrations in pregnant women can rise to ~731 nM [3]. If P4 concentration is below the normal level, people need to take P4 supplements. However, when the concentration of P4 is over the normal level, P4 can be toxic and have carcinogenic effects [4]. So it is crucial to monitor and control P4 concentration in an appropriate range. Yet, P4 detection in biological matrices is challenging since the concentrations are around the $ng \cdot mL^{-1}$ ($1\ ng \cdot mL^{-1} = 0.325$ nM) level or even lower [5]. Thus, more sensitive and effective assay is required.

Although many analytical methods have been developed to monitor P4, there are only two kinds of methods commonly used which is immunoassays (radioimmunoassay, enzyme-linked immunosorbent assay, electrochemical immunoassay, etc.) and chromatographic methods (high performance liquid chromatography, liquid chromatography-mass spectrometry, gas chromatography-mass spectrometry et al.) [6,7]. These methods are sensitive but they require expensive equipments and complicated performance procedures, or some suffer from poor specificity, accuracy and reproducibility because of the cross-reaction and batch-to-batch variation of antibodies [8]. Aptamer was first selected and named in 1990 [9,10]. Since then, aptamers of various targets (metal ions, proteins, antibiotics, etc.) have been selected and applied to their target detection [11-20]. There is intermolecular interaction between the aptamer and its target so that they can bind to each other with high selectivity and affinity [14]. Thus, aptamers have been applied to clinical, medical monitoring and food analysis [21]. Rencently, some researchers successfully selected P4 aptamer and developed an electrochemical aptasensor with a detection limit of 2.86 nM [12]. And based on this breakthrough, our group had built a NaCl-induced colorimetric aptasensor which obtained a LOD of 2.62 nM [22].

Gold nanoparticle (AuNP) is superior material for signal transduction due to its aggregation behavior and has been frequently used in the design of biosensors [23-28]. AuNPs alone are dispersed and appear red in solution. The aggregation

of AuNPs will bring about an increase in the size of particles in solution, leading to a rise in the absorbance within a certain range and a color change to blue.

Hexadecyltrimethylammonium bromide (CTAB) possesses two effective features in this biosensor: CTAB can not only aggregate AuNPs but also bind to DNA, owing to its positive charge and the electrostatic attraction [29,30,31]. As a cationic surfactant, CTAB has also been applied in the isolation of genomic DNA from plants samples. Futhermore, the binding of CTAB to ssDNA and dsDNA are different: ssDNA-CTAB complexes are formed with cubic nanostructures, while dsDNA forms hexagonal nanostructures with CTAB [23,32]. The principle of the colorimetric biosensor under ideal conditions was outlined in **Scheme 1**. In the absence of P4, P4 aptamers are free to form some supramolecule with CTAB, so that the AuNPs maintain dispersed. However, the sensing solution will turn from red to blue in the presence of P4 because P4 aptamer are used up firstly owing to the formation of an aptamer-P4 complex, leaving CTAB free to cause the aggregation of AuNPs. Besides, the absorbance ratio $A650/A520$ has a linear correlation with the concentration of P4 in a certain range. Therefore, this assay makes it possible to detect P4 in biological fluids not only by absorbance measurement but also through naked eyes.



Scheme 1 Schematic diagram of the colorimetric aptasensor for progesterone (P4) detection based on hexadecyltrimethylammonium bromide (CTAB)-induced aggregation of AuNPs.

2. Materials and Methods

2.1 Chemicals and materials

CTAB, diethylstilbestrol (DES), ampicillin (AMP), thiamphenine (THI), aztreonam (AZT), were obtained from Sigma-Aldrich (St. Louis, MO, USA; www.sigmaaldrich.com). P4, 17β-estradiol (E2), estriol (E3), bisphenol A (BPA), sodium citrate, $HAuCl_4 \cdot 4H_2O$, NaOH, 3-(N-Morpholino) propanesulfonic acid (MOPS, 10 mM, pH 8.0), were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China; shreagent.lookchem.com). Synthetic human urine and serum were purchased from BIOMART.CN (www.biomart.cn). Unless otherwise mentioned, all other reagents were of analytical reagent grade and used without further purification. The 96-well microplates and centrifuge tubes were bought from Thermo Fisher Scientific

Inc. (Nunclon, Denmark; www.thermofisher.com/cn/zh/home.html). The ultrapure water used in all experiments and aqueous solution preparation was purified by a Milli-Q Advantage A10 System (Millipore Inc., Bedford, MA, USA). The sequence of P4 aptamer is 5′-GCATCACACACCGATACTCACCCGCCTGATTAACATTAGCCCACCG CCCACCCCCGCTGC-3′ (60-mer) [12], synthesized by Sangon Biotechnology Inc. (Shanghai, China; www.sangon.com). The aptamer was dissolved in ultrapure water and stored at 4 °C before use.

2.2 Instrumentation

Colorimetric assays were recorded on an Infinite M200 Pro microplate spectrophotometer (Tecan Austria GmbH, Salzburg, Austria). A thermostat (Eppendorf, Hamburg, Germany) was used for incubation. A circular dichroism spectroscopy (CD; J-815, Jasco, Japan) was employed to characterize the structural changes of P4 aptamer. The average diameter of AuNPs was measured on a Zetasizer Nano S (Malvern, England).

2.3 Preparation of AuNPs

AuNPs were prepared by sodium citrate reduction of $HAuCl_4 \cdot 4H_2O$, based on a previous study [24]. First, the boiled solution of $HAuCl_4 \cdot 4H_2O$ (100 mL, 0.03% (w/w) was added with 3.5 mL of 1 % (w/v) sodium citrate solution, then continue to be boiled and stirred for 30 min. Within that period, the color of solution changed gradually from light grey, blue, purple, and to wine red finally. The mixture was boiled for another 10 min and stirred until it was cooled to

room temperature. At last, the cooled solution was filtered by 0.2 μm ultrafiltration membranes (HP134, Sangon Biotechnology Inc.), and then stored in a brown glass bottle at 4 °C for further use.

2.4 Characterization of the interactions among aptamer, P4, CTAB and AuNPs

The CD spectrum of 4 samples was tested: (a) 200 nM aptamer; (b) 200 nM aptamer + 200 nM P4; (c) 200 nM aptamer + 40 μM CTAB; (d) 200 nM aptamer + 200 nM P4 + 40 μM CTAB. Sample (a) was directly tested, sample (b) and (c) were tested after 20-min incubation. As for sample (d), CTAB was added after 20-min incubation of the aptamer and P4, then the CD spectrum was tested after another 20 mins.

The dynamic light scattering (DLS) is to measure the average diameter of AuNPs in 4 different samples: (a) AuNPs; (b) AuNPs + 2.6 μM CTAB; (c) Sample b + 28 nM aptamer; (d) Sample c + 2 μM P4. Sample (a) was directly tested and sample (b) were measured after 20-min incubation. As for sample (c), AuNPs was added after 20-min incubation of the aptamer and CTAB, then the particle size of AuNPs was measured after another 10 mins. Aptamers and P4 were firstly incubated for 20 mins in sample (d), then CTAB was added for another 20-min incubation, following the 10-min incubation with AuNPs. At last, the size of AuNPs was measured.

2.5 Measurement of the absorbance

At the end of each experiment, the absorbance values at 650 nm (*A*650) and

520 nm (*A*520) were recorded. Then the absorbance ratio A = A650/A520 was calculated to reflect the aggregation degree of AuNPs.[23,25,33]. In some experiments, the *A*650/*A*520 values in the blank solutions without P4 (A_0) and sample solutions containing P4 (*A*) were both measured to calculate the values of ΔA ($\Delta A = A_0$-*A*).

2.6 Optimization of detection conditions

The total volume of each sensing system was set at 500 μL, containing 200 μL AuNPs. P4 was firstly dissolved to 1 mM in anhydrous ethanol, which was diluted to the desired concentration of P4 by MOPS buffer. Unless otherwise noted, all experiments were performed at 25 °C. And all solutions were mixed sufficiently by pipette. Each experiment was performed for three times.

To optimize the concentration of CTAB, 200 μL AuNPs were treated with different volumes of 20 μM CTAB solution (25, 35, 45, 50, 55, 60, 65, 70, 75, 85, 95 and 105 μL) and MOPS buffer were added to obtain a total volume of 500 μL. After 10-min incubation, *A*650 and *A*520 were recorded, and the minimum CTAB concentration that can fully lead to the aggregation of AuNPs was chosen.

For the optimization of P4 aptamer concentration, MOPS buffer with 0.2 μM P4 aptamer at different volumes (10, 20, 30, 40, 50, 55, 60, 65, 70, 80 and 90 μL) were incubated for 20 min with or without 2 μM P4 respectively, as the experimental and blank groups. Then, all groups were added with the optimized concentration of CTAB. After 20 mins, 200 μL AuNPs was added and

cultivated for 10 min. Ten mins later, A_0, A and ΔA were measured, and the minimum concentration of aptamer resulting in maximum ΔA was chosen.

2.7 Sensitivity and selectivity of this aptasensor

For the sensitivity investigation, optimized concentration of P4 aptamer and P4 with various final concentrations (0, 10, 20, 50, 100, 200, 300, 400, 500, 600, 800, 1000 nM) were added in centrifugation tubes with MOPS buffer. After 20-min incubation, the optimized concentration of CTAB was added to incubate for another 20 min. Then, 200 μL AuNPs was added for 10-min incubation. Then, A_0, A and ΔA were measured.

In order to test the selectivity, 2 μM potential interferents such as E2, E3, DES, AMP, THI, AZT, BPA and the mixture (P4 + E2 + E3) were added to the biosensor instead of P4 in the sensitivity testing. Finally, A_0, A and ΔA were recorded.

2.8 Application in urine and serum samples

According to previous reports [20,23], the sensing system was established in the ideal buffered condition, and then applied to urine and serum for P4 detection. Synthetic human urine and serum were firstly diluted by ultrapure water according to the previous report [19]. Then, different concentrations of P4 (50 nM, 100 nM, 300 nM, 500 nM) was spiked to the human serum and urine samples respectively to investigate the practicability of the assay. Then they were tested by the established biosensor.

3. Results and Discussion

3.1 Interactions among aptamer, P4, CTAB and AuNPs

CD spectrum can reflect the structural change of P4 aptamers effectively. According to the principle of this assay, both P4 and CTAB are able to interact with the P4 aptamer to form complexes. Therefore, CD was used to monitor the structural change of P4 aptamers. As shown in **Fig. 1**, the P4 aptamer had a negative peak at 240–260 nm and a positive peak at 270–290 nm, which corresponds to B-DNA structure [34]. After the addition of P4 or CTAB or both P4 and CTAB, the bands intensity changed, which indicated a disruption of staking contact of the bases and a destabilization of the DNA helix [35]. Therefore, it could be concluded that the aptamer (ssDNA) interacted with P4 or CTAB through an intercalation mode and changed into a different conformation [35].

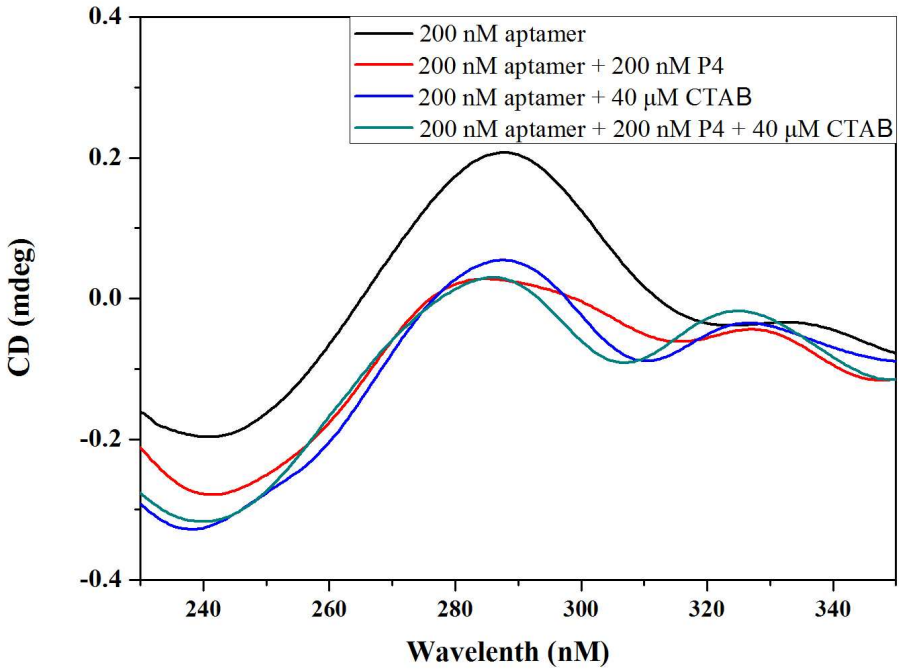


Fig. 1 Circular dichroism (CD) spectra of progesterone (P4) aptamer treated with P4 or hexadecyltrimethylammonium bromide (CTAB).

DLS was employed to characterise the aggregation behavior of AuNP and confirm the principle of this biosensor. **Fig. 2** shows the AuNPs distribution of different size treated with various substances. The original AuNPs were well dispersed in spherical shape with an average diameter of 11 nm (**Fig. 2a**). After the addition of CTAB, due to the formation of the CTAB-AuNPs supermolecular complex, significant aggregation of AuNPs was observed (**Fig. 2b**). In the absence of P4, CTAB was exhausted by the aptamers so that the AuNPs were still dispersed (**Fig. 2c**). After adding P4, AuNPs aggregated into larger clusters since the aptamers binded to P4, leaving CTAB to aggregate AuNPs (**Fig. 2d**).

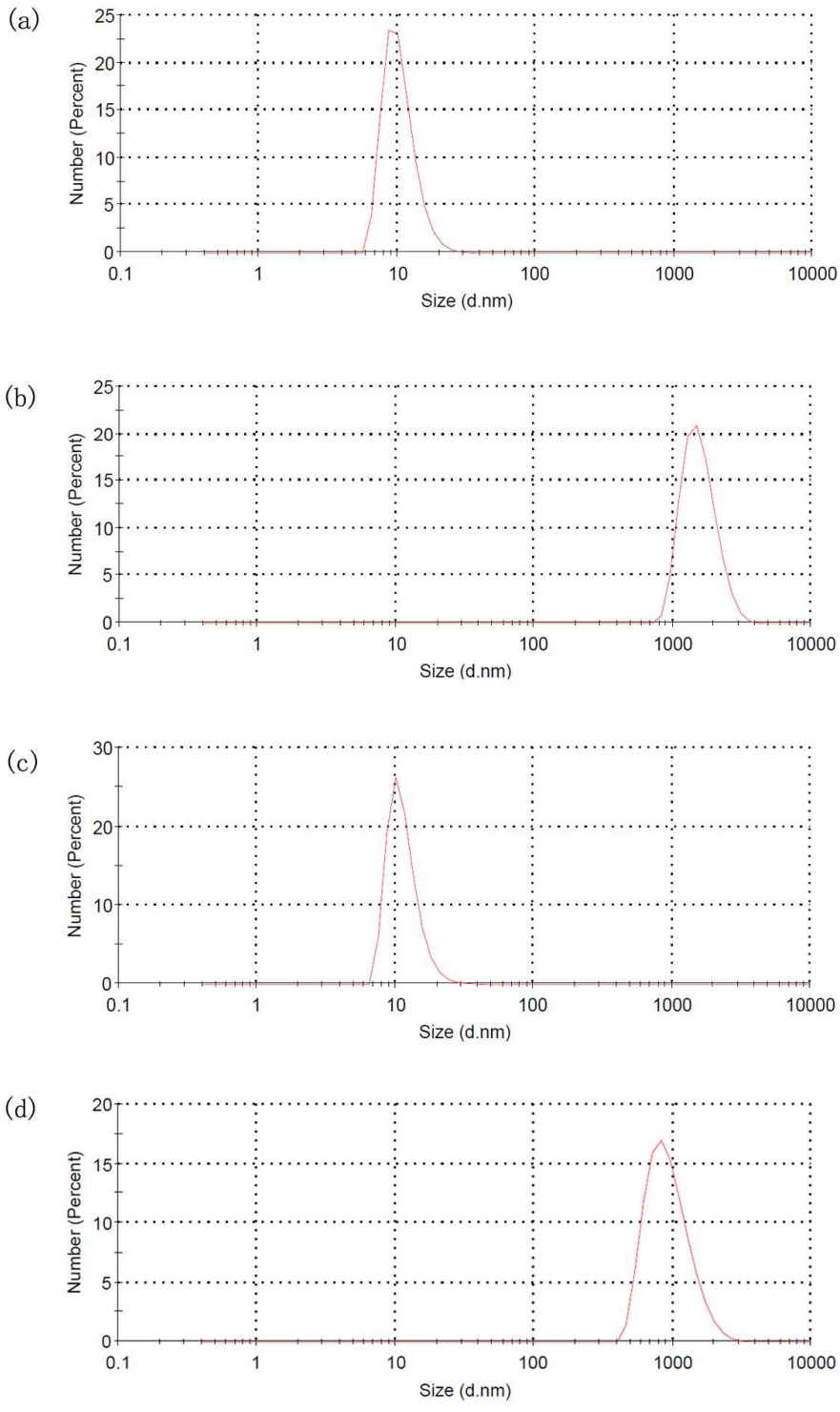


Fig. 2 Particle size changes of gold nanoparticle (AuNP) solutions in the presence of different substances: (a) AuNPs; (b) AuNPs + 2.6 μM CTAB; (c) Sample b + 28 nM aptamer; (d) Sample c + 2 μM P4.

3.2 Optimization of detection conditions

The following parameters were optimized: (a) CTAB concentration; (b) aptamer concentration. Respective data and Figures are given in the Supplementary Material. The following experimental conditions were found to give best results: (a) a 2.6 μM concentration of CTAB (**Fig. S1**), (b) a P4 aptamer concentration of 26 nM (**Fig. S2**).

3.3 Sensitivity of this biosensor

The colorimetric biosensor was carried out with concentrations of P4 from 0 to 1000 nM, and the absorbance values at 520 and 650nM were recorded. As shown in **Fig. 3**, AuNPs aggregated gradually with increasing concentrations of P4. Meanwhile, the color of the sensing solution gradually changed from wine red, purple to blue. The presence of P4 is distinguishable with naked eyes when the concentration of P4 is above 20 nM. In addition, the ΔA at low P4 concentrations was fitted to a linear plot with a regression equation of $\Delta A = 0.0014\ C_{P4} + 0.0398$ (R = 0.99). Referring to previous reports [23,25], $3\sigma/s$ represents the limit of detection (LOD), where $\sigma = 3.84\times10^{-4}$ is the standard deviation of the instrument that was calculated from the measurements of the blank sample (**Table S1**) and s =0.0014 is the slope of the linear calibration plot. So the detection limit is 0.89 nM, which is generally lower than the P4 concentration in serum and urine of people in most cases. Futhermore, $A650/A520$ has a linear correlation with the P4 concentration of 0.89–500 nM. Compared with other P4 detection methods (**Table 1**), this aptasensor offers a

number of advantages, including high sensitivity, convenience and visual detection. Although chromatographic methods are sensitive, accurate and reproducible, these require expensive instruments and intensive labor. Immunoassay methods are widely used in clinically determination but they also have drawbacks like time-consuming, poor specificity or reproducibility.

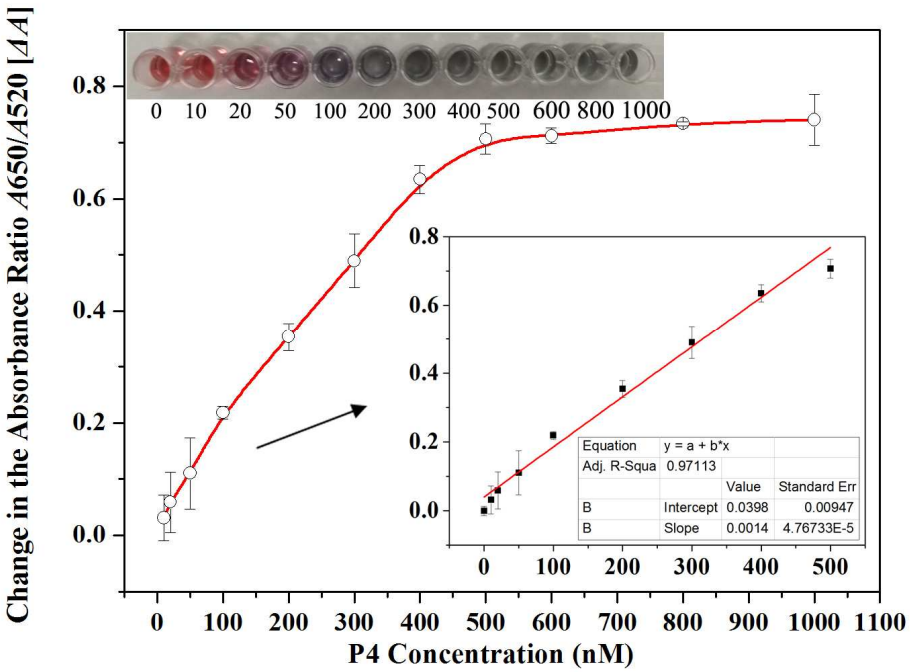


Fig. 3 Sensitivity of the biosensor for progesterone (P4) detection. The picture displays the absorbance ratio increase of the sensing solutions treated with 0, 10, 20, 50, 100, 200, 300, 400, 500, 600, 800 and 1000 nM P4. The inset shows the linear response at low concentrations of P4.

Table 1 Comparison between our aptasensor and previous methods for progesterone (P4) detection

Method	Matrix	LOD (nM)	Linear range (nM)	Ref.

HPLC-DAD	Urine	1.49	30.2–3021	[36]
HPLC-UV	Urine	0.22	0.32–636	[37]
SPE-LC-MS/MS	Serum	0.10	0.25–79.5	[38]
CdSe/ZnS quantum dot-based immunoassay	Serum	0.67	1.24–14.5	[2]
impedimetric aptasensor	Water	2.86	31.8–292	[12]
NaCl-induced colorimetric aptasensor	Urine	2.62	2.62–600	[22]
CTAB-induced colorimetric aptasensor	Serum, urine	0.89	0.89–500	This work

HPLC-DAD: high performance liquid chromatography-diode array detection. UV: ultraviolet. SPE-LC-MS/MS: Solid phase extraction- liquid chromatography-mass spectrometry/ mass spectrometry. GC-MS: gas chromatography-mass spectrometry. LOD: limit of detection. CTAB: hexadecyltrimethylammonium bromide.

3.4 Selectivity of this biosensor

To conform the selectivity of this assay, potential interferents (E2, E3, DES, AMP, THI, AZT, BPA) and the mixture solution (P4+E2+E3) were also tested respectively. The results in **Fig. 4** indicate that this aptasensor possesses good selectivity since ΔA of the solution containing P4 is much higher than others. And the interference of other chemicals is slight and neglectable.

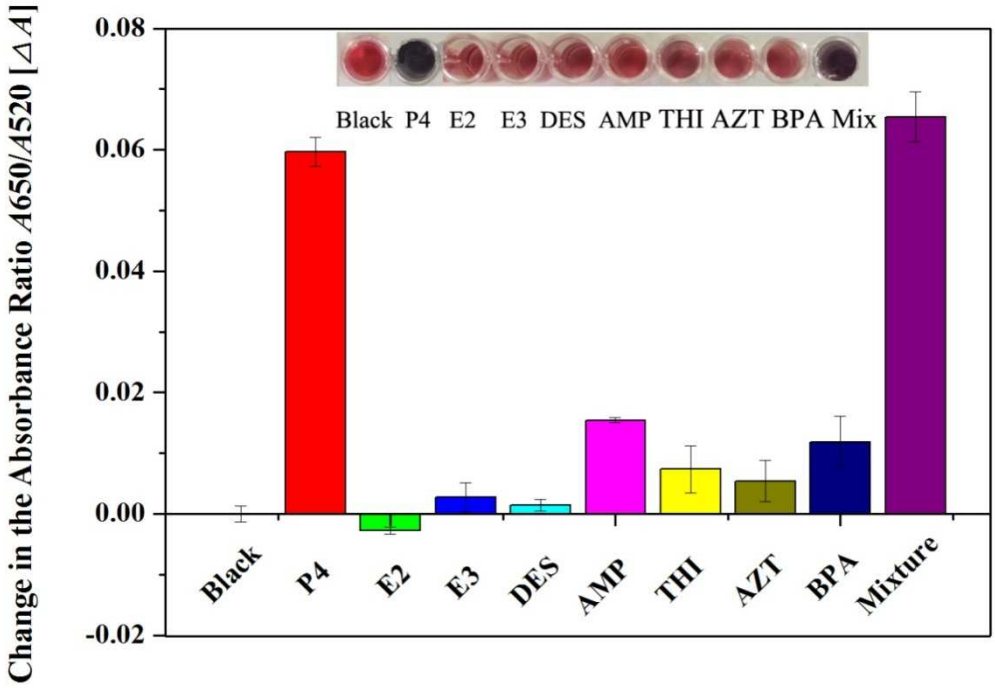


Fig. 4 Selectivity of the biosensor for progesterone (P4) detection using CTAB to aggregate gold nanoparticles (AuNPs). Experimental conditions: 200 μL AuNPs, 26 nM aptamer, 2.6 μM hexadecyltrimethylammonium bromide (CTAB), 3-(N-Morpholino) propanesulfonic acid (MOPS) buffer (10 mM, pH 8.0) and 2 μM progesterone (P4), 17*β*-estradiol (E2), estriol (E3), diethylstilbestrol (DES), ampicillin (AMP), thiamphenicol (THI), aztreonam (AZT), bisphenol A (BPA), the mixture (P4 + E2 + E3).

3.5 Detection of P4 in serum and urine samples

This aptasensor was employed in human serum and urine to investigate practical applicability. The results were showed in **Table 2** and **Table 3**: the recoveries for P4 at all concentrations were 102.4–116.5 % and 93.2–107.6 %,

the relative standard deviations (RSD) were in the range of 2.8–6.5 % and 3.4–5.5 %. The good recovery of this aptasensor indicates that there is no significant interference from urine or serum components in the aptasensor for P4 detection, suggesting a great potential for P4 detection in biological fluids.

Table 2 Determination of progesterone (P4) in serum samples

Spiked Concentration (nM)	Found Concentration (nM)	Recovery (%)	RSD (%)
50	51.2	102.4	3.5
100	116.5	116.5	6.5
300	315.4	105.1	2.8
500	518.9	103.7	4.2

Table 3 Determination of progesterone (P4) in urine samples

Spiked Concentration (nM)	Found Concentration (nM)	Recovery (%)	RSD (%)
50	53.8	107.6	5.5
100	93.2	93.2	3.8
300	307.8	102.6	3.4
500	486.5	97.3	4.6

4. Conclusions

A colorimetric aptasensor for P4 detection has been successfully developed based on the aggregation of AuNPs controlled by the interactions among P4, P4

aptamer and CTAB, with high selectivity and a detection limit of 0.89 nM. Besides, P4 in the range of 0.89–500 nM are detectable via absorbance measurement and the presence of P4 can also be observed by naked eyes when the concentration is above 20 nM. Due to the good sensitivity and recovery of this aptasensor, its application in biological fluids for P4 detection is promising and worthy of further study.

5. Acknowledgements

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