

Using Simple-Structured Split Aptamer for Gold Nanoparticle-based Colorimetric Detection of Estradiol

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Demand for the detection of estradiol, which is a naturally occurring hormone, has been increasing. Gold nanoparticle-based colorimetric aptasensors have been developed for estradiol detection; however, the long sequence of aptamers due to the formation of the secondary structure likely affects the sensitivity of the aptasensors. Herein, a sensitive colorimetric biosensor is developed for label-free detection of estradiol by using an estradiol-specific split aptamer. The results demonstrate that a superior response is observed when a split aptamer with a high free energy of the secondary structure ($\Delta G > -3$ kcal/mol) is used, in comparison to that observed using a split aptamer with a low free energy of the secondary structure ($\Delta G < -3$ kcal/mol) at 27°C. After selecting the appropriate split aptamer, the standard calibration curve obtained for estradiol has a detection limit of 6.7 nM, with a linear range of 6.7 nM – 66.7 μ M in the logarithmic scale. Furthermore, this assay is sensitive, easy-to-operate, inexpensive, and non-time-consuming (provides results within 50 min), thereby showing potential for clinical applications (detection of other small molecular targets).

Keywords Split aptamer, colorimetric assay, gold nanoparticle, estradiol detection, localized surface plasmon resonance

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Introduction

Estradiol is a naturally occurring hormone in humans and animals with strong estrogenic effects, including stimulating uterine growth, maturing germ cells, and maintaining healthy pregnancy.¹ It also plays a role in reducing blood cholesterol by regulating the distribution of body fat and in preventing bone loss by maintaining bone and joint health. The residues of estradiol in animal products such as milk can be harmful to humans. High concentrations of estradiol are associated with increased breast cancer risk and may cause liver damage. Elevated estradiol levels in men are indicative of the presence of feminine sexual characteristics, such as male breast development.² Additionally, previous studies have reported that estradiol can negatively influence the proliferation and migration of newborn cells.^{3,4} Hence, it is vital to detect the presence and measure the concentration of estradiol in environmental and clinical samples. Thus, the development of a rapid, sensitive, and specific detection method for estradiol is essential.

Although immunological techniques and instrumental analytical approaches are available for the detection of estradiol, these are generally expensive and complicated and require highly trained personnel.^{5,6} Furthermore, the use of antibodies for target recognition leads to disadvantages in terms of the high cost of production and their stability.⁷ To overcome these drawbacks, nucleic acid-based aptamer probes have been widely used to design various types of fluorescence, electrochemical,

surface plasmonic resonance, or colorimetric sensing techniques.^{8–12} Among these, gold nanoparticle (AuNP)-based methods have gained considerable interest because of their simple and well-established synthesis procedures and notable optical effects that allow a colorimetric read-out of the binding event.^{13–15} Linear single-strand DNA (ssDNA) aptamers are easily attached onto the surface of AuNPs through a coordination interaction between the Au surface and N atoms of the DNA nucleobases. Conversely, folded ssDNA aptamers (such as hairpin and pseudoknot structures) or double-strand DNA (dsDNA) are unfavorable primarily due to their rigid secondary structures;^{16–18} N atoms are located inside the double-stranded DNA and therefore, are unavailable for attachment. Hence, linear ssDNA aptamers protect AuNPs from salt-induced aggregation, but in the presence of targets, the aptamers are folded by binding to the targets and being desorbed from the surface of the AuNPs, resulting in subsequent aggregation of AuNPs and a change in the color of the solution from red to purple-blue. The combination of an ssDNA aptamer and AuNPs can allow for a simple and direct detection of target molecules; this approach has been extensively employed in various analysis techniques.^{19–21} Nevertheless, several DNA aptamers, such as the estradiol aptamer with secondary structures, cannot electrostatically stabilize AuNPs, which increases the difficulty and complexity of colorimetric detection.

To overcome this limitation, aptamers can be split into two fragments that stay separate in the absence of a target but assemble in the presence of the target. It has been reported that split aptamers, in contrast to non-split aptamers, retain their affinity and specificity and also enhance the sensitivity of an assay.^{22–24} This result supports the development of a highly

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Table 1 Sequence and folding free energies of the studied estradiol-binding aptamers

Split aptamer probe	Sequence (5' - 3')	Free energy of secondary structure/ kcal mol ⁻¹
A1	AAGGGATGCCGTTTG	-0.58
A2	GGCCCAAGTTCGGCATAGTG	-2.54
B1	AAGGGATGCCGTTTG	-0.58
B2	GCCCAAGTTCGGCATAGTG	-1.87
C1	AAGGGATGCCGTTTGGG	-1.13
C2	CCCAAGTTCGGCATAGTG	0
D1	AAGGGATGCCGTTTGGGC	-3.04
D2	CCAAGTTCGGCATAGTG	0
E1	AAGGGATGCCGTTTGGGCC	-3.42
E2	CAAGTTCGGCATAGTG	0
35-mer aptamer	AAGGGATGCCGTTTGGG	-9.12
	CCCAAGTTCGGCATAGTG	

sensitive AuNP-based colorimetric aptasensor by using long-sequence aptamers. However, the selection criterion for splitting an aptamer into two fragments has not been investigated thoroughly. Herein, we develop a colorimetric split aptamer-based biosensor and investigate the sensitivity of split aptamers with different free energies of their secondary structure. This method was used to detect estradiol in synthetic urine samples in order to verify its performance.

Experimental

Reagents and chemicals

The sequences of the estradiol aptamer²⁵ and the split aptamer fragments were synthesized and purified by Purigo Biotech, Inc. (Taipei, Taiwan), as shown in Table 1. Estradiol, ethinylestradiol, 1-aminoanthraquinone, 2-methoxynaphthalene, estrone, testosterone, and sodium chloride were obtained from Sigma-Aldrich (St. Louis, MO). Sigmatrix urine diluent, which mimics human urine, was also obtained from Sigma-Aldrich. Next, 20-nm AuNPs were purchased from BBI Solution (Madison, WI). All chemicals were of the analytical grade, and solutions were prepared using milliQ water (Millipore, Bedford, MA).

Calculating free energy of secondary structure formation of split aptamers

The folding free energy for the split sequences was calculated using NUPACK (<http://www.nupack.org/>).²⁶ The sequences were folded at 27°C using the default settings with the secondary structure models (Table 1).

Detection of estradiol using split aptamer

Prior to the measurement, the DNA solutions were heated to 90°C for 5 min and then snap-cooled on ice for 2 min to avoid the formation of DNA duplexes and to promote the conformer formation of ssDNA. First, 2 µL of 1.5 µM long-sequence estradiol aptamer or 2 µL of 1.5 µM split aptamers [C1 and C2] was incubated with 100 µL of the AuNP solution for 10 min. Then, 10 µL of estradiol solutions with different concentrations were added. Next, 36 µL of 1X TE buffer was added to the solution to attain a final volume of 148 µL. The mixture was maintained at room temperature for an additional 10 min. Subsequently, 2 µL of 4 M NaCl was added to this mixture. After the solution was equilibrated for 25 min, the resulting solution was characterized using UV-vis spectroscopy. The

spectrum was monitored over wavelengths ranging from 400 to 800 nm, and the photographs were captured using an iPhone 7 smartphone.

Different interferents, including ethinylestradiol, diethylstilbestrol, bis(4-hydroxyphenyl) methane, estrone, and testosterone, were used for testing the specificity of this assay; their concentration was kept constant at 6.7 µM. The AuNP solutions were first reacted with 1.5 µM split aptamers of C1 and C2. Then, 10 µL of the interfering substance and 36 µL of 1X TE buffer were added, and the mixture was incubated for 10 min. Next, 2 µL of 4 M NaCl solution was added to the above-mentioned reaction mixture. The mixed solution was incubated for 25 min and then characterized using UV-vis spectroscopy.

Results and Discussion

It is considered that electrostatic repulsion between core particles is responsible for AuNP dispersion in aqueous medium and, subsequently, stable under physiological conditions. In addition, the van der Waals interaction is responsible for AuNP aggregation as an attractive force.^{27,28} As shown in Fig. 1(A), the 35-mer estradiol aptamer was designed into two split aptamer fragments [C1 and C2], which formed simpler structures and could be easily attached on the surface of AuNPs to protect gold colloids because of electrostatic forces. The presence of DNA aptamer fragments helped to inhibit the aggregation of AuNPs caused by van der Waals interactions.²⁹ With the addition of estradiol, C1 and C2 were released from nanoparticle surfaces to assemble into a stable complex upon interacting with the target. Thus, AuNPs become unstable when they lose their surface charges, leading to the aggregation of AuNPs and a change in the color of the solution from red to blue when adding a high concentration of salt. We began to apply the previously reported 35-mer estradiol aptamer²⁵ for the AuNP aggregation assay (Fig. 1(B)). Without the DNA aptamer, the free AuNPs exhibited a sharp surface plasmon peak at 660 nm due to the aggregation of AuNPs (Fig. 1(B)c). When the aptamer was added, a new peak appeared at 520 nm, indicating that the AuNPs did not aggregate (Fig. 1(B)b). However, an evident color change occurred when 666.7 µM of estradiol was added, and the absorption intensity increased to 650 nm (Fig. 1(B)a). Hence, the ratio of the absorbance at 650 and 520 nm (A650/A520) can help provide an understanding of the degree of aggregation and the color change of AuNPs (Fig. 1(C)). Using split fragments with the same parameters [(the length of aptamers) × (the concentration of aptamers)] resulted in discriminable color difference in the presence and absence of the target, in comparison to that observed using a non-split aptamer (Figs. 1(D)a and 1(D)b). Even under the same total concentration of aptamers (66.7 nM), the solution with split aptamer probes [C1/C2] was a deeper shade of red than that with original aptamers (Figs. 1(D)c and 1(D)d). The absorbance ratio (A650/A520) with the target was then divided by the absorbance ratio without the target (as a blank control) to obtain the signal-to-blank ratio (SBR). The relationship between the SBRs and split aptamers is shown in Fig. 2(C). Although the aggregation responses of both split aptamers (Fig. 1(E)) were lower than that of the 35-mer aptamer, SBRs of both the fragments were greater than or equal to that obtained using the 35-mer aptamer, implying that the split aptamer might effectively stabilize AuNPs.

Next, we investigated the position of the splitting site in the loop in order to realize the optimal interaction and signal transduction (Fig. 2(A)). From Table 1, free energy is 0, which

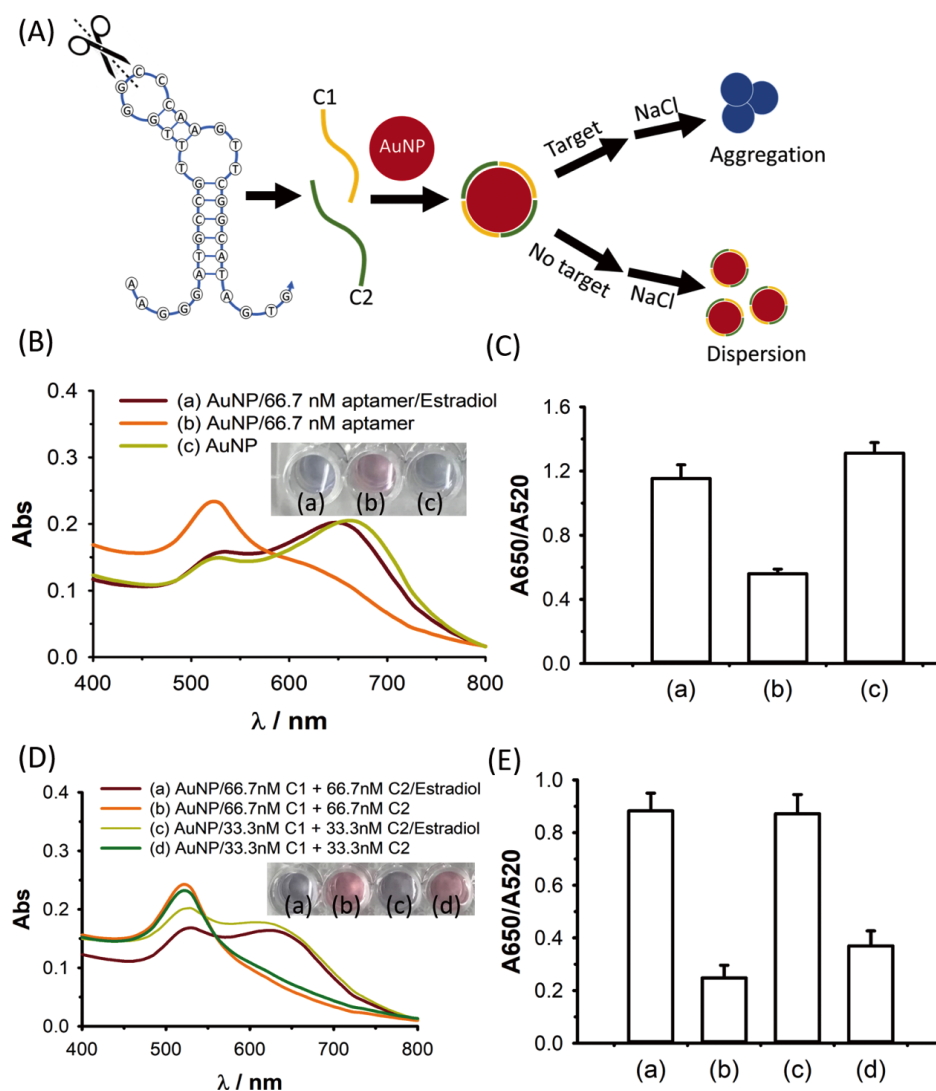


Fig. 1 (A) Schematic of colorimetric detection method for estradiol based on split aptamer recognition. (B) Absorption spectra and (C) ratio of absorbance at 650 and 520 nm for AuNP solutions in the presence and absence of 666.7 μM estradiol obtained using the 35-mer aptamer. (D) Absorption spectra and (E) ratio of absorbance obtained using the split aptamer. The average number of aptamers on each nanoparticle was estimated at around 13 when 66.7 nM aptamer probes were present in the AuNP solutions. The error bars represent the standard deviation of the mean of three measurements.

is favorable for the linear DNA formation. The lower the free energy, the higher the stability of the secondary structure. Compared with original aptamers, split fragments with less secondary structures are more useful for binding.³⁰ Namely, split aptamers with more secondary structures are unfavorable for target binding, thus reducing the effective recognition of estradiol. We observed that the lower free energy of the secondary structure in one strand of split aptamers (such as D1 and E1 probes), led to a weaker response in the presence of estradiol (Fig. 2(B)). This is probably due to the generation of an unfavorable folding structure of the split DNA strand; the interaction of an estradiol target would likely compete poorly with the free energy required to open this folding structure. In addition, the best SBR is obtained when both split DNA fragments have higher free energy of the secondary structure formation (such as C1/C2), presumably because these split fragments with less secondary structures, which easily enables the target-induced association of the split fragments, produce a larger signal response. Another possible reason is that, when

DNA is adsorbed on the surface of AuNPs, the structured aptamer binds more tightly than linear DNA; thus, the split DNA structure desorbs more slowly, leading to a lower signal response. Interestingly, we found that the SBR of split fragments in E1/E2 and D1/D2 was almost the same as that obtained using the 35-mer aptamer, indicating the importance of choosing an appropriate split position (Fig. 2(C)). Considering the results of SBR, a split aptamer [C1/C2] with a putatively less complex DNA structure was selected for the following study.

The performance of the nanoplasmonic aptamer biosensor was investigated using a split aptamer [C1/C2]. The ratio of A_{650}/A_{520} was plotted against the log of estradiol concentration. The absorption ratio gradually increases with an increase in the estradiol concentration for the split aptamer, while the ratio reaches a plateau at an estradiol concentration of more than 66.7 μM (Fig. 3(A)). The limit of detection (LOD) of the 35-mer aptamer was 6.7 nM, with a dynamic range from 0.67 to 66.7 μM . Under the same conditions, we obtained the same LOD by using the split aptamer, but we were able to achieve a

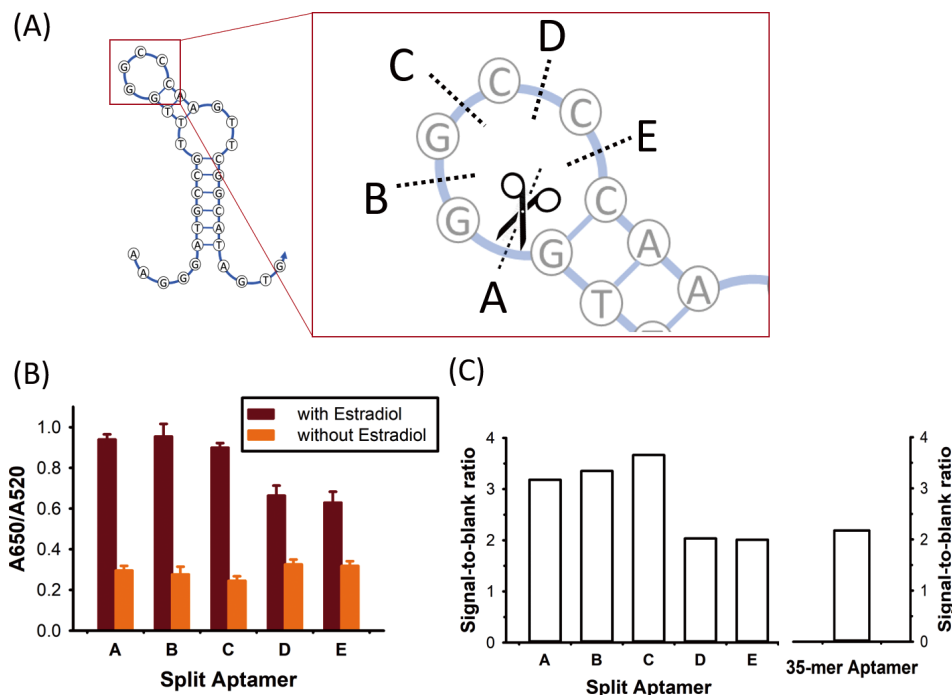


Fig. 2 (A) Secondary structure of the 35-mer aptamer predicted using NUPACK. The black dotted line indicates the splitting site. (B) The ratio (A_{650}/A_{520}) in the presence and absence of estradiol with different split sites. (C) Comparison of the signal-to-blank ratios of different split fragments and the original 35-mer aptamer.

significantly wider dynamic range compared to that observed using the 35-mer aptamer. As shown in Fig. 3(A), the LOD of the split aptamer [C1/C2] was 6.7 nM, but the dynamic range was from 6.7 nM to 66.7 μ M. Our linear dynamic range is at least two orders of magnitude larger than that obtained using the original 35-mer aptamer.

Then, we explore the effect of a single split aptamer fragment (C1 or C2) on estradiol detection (Fig. 3(B)). As expected, C1 or C2 was still responsive to estradiol because of the partial binding capacity of the 35-mer aptamer; however, in terms of sensor performance, such as LOD and linear range, the results obtained using C1 and C2 separately were worse than those obtained when they were used together. In addition, we explored the effect of the split aptamer [E1/E2] with different thermodynamic energies of its structure. As observed in Fig. 3(C), although split fragment sequences originated from the 35-mer aptamer, the sensitivity for estradiol was greatly different over split aptamers. Compared to that obtained using a split aptamer [C1/C2] with a putatively less folding structure, a split aptamer [E1/E2] with a lower free energy of the structure provided lower LOD and a narrower dynamic range. In this case, we found that the sensing performance of the split aptamer [E1/E2] was slightly lower than that of the original aptamer, presumably owing to the low affinity of the split aptamer to the target. Importantly, the results showed that selecting an inappropriate split fragment would result in a reduction in the overall performance of the assay.

The specificity of this assay for estradiol was estimated by testing other possible contaminating agents with structures similar to that of estradiol (E2), such as ethinylestradiol (EE), 1-aminoanthraquinone (1AQ), 2-methoxynaphthalene (2MN), estrone (E1), and testosterone (TS). The results indicated that only estradiol can cause an evident change in signal values, which is accompanied by a change in color from red to purple

(Fig. 4), whereas the addition of other interfering molecules at concentrations of 6.7 μ M to the split probes and AuNP solution showed no notable color change. It is noted that there was a slight increase in the absorption ratio in the presence of testosterone, which may suggest that the affinity of the original aptamer is slightly affected by testosterone (Fig. S1, Supporting Information).

The assay was further employed for the practical application test by using synthetic urine, which mimicked human urine in terms of composition. The spiked samples were prepared by adding an estradiol standard solution to an appropriate diluted solution of synthetic urine (Fig. S2, Supporting Information). As shown in Fig. 5(A), the change in the absorption ratios exhibits the same trend as seen in a pure buffer, showing an increase with the estradiol concentration. Furthermore, the responses to estradiol in the buffer were well correlated with those from the spiked urine samples, showing a good linear relationship with a correlation coefficient (R^2) of 0.98. In this study, the LOD in the urine sample was reported to be 6.7 nM. Nevertheless, a 500-fold dilution of the urine sample is required before measurement; accordingly, the LOD should be multiplied by 500-fold, which amounts to ~ 3.4 μ M for practical applications. Consequently, further efforts for practical applications are required to reduce the matrix effect by introducing anti-fouling materials³¹ or to enhance the performance of the assay by using detectors³² that are more sensitive.

Conclusions

This work demonstrates a sensitive colorimetric estradiol detection method that is based on a target-induced assembly of split aptamers. We find that varying the split site of the loop on

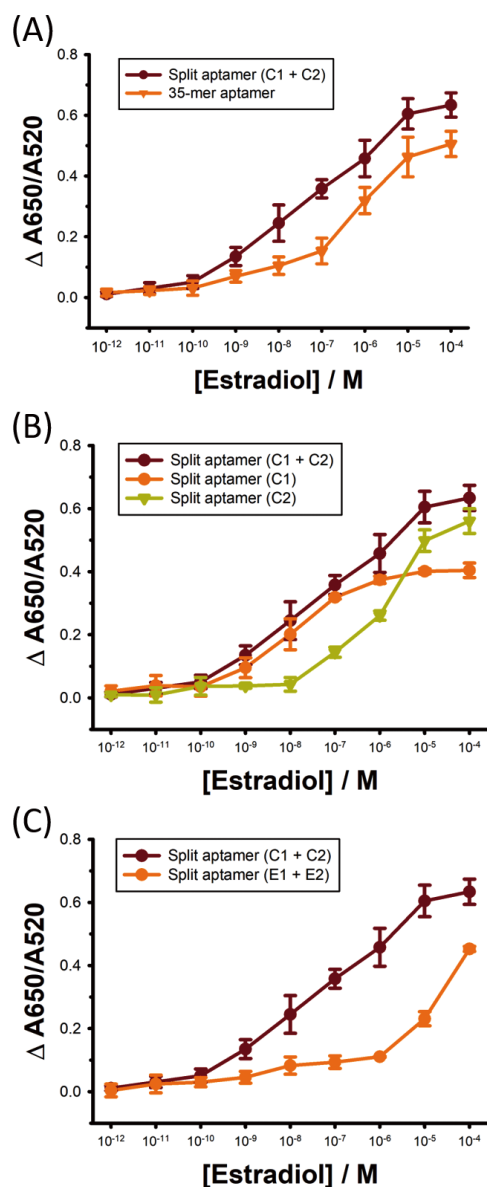


Fig. 3 Signal response ($\Delta A_{650}/A_{520}$) toward a range of estradiol concentrations using the split aptamer [C1 + C2] compared with that obtained using the (A) original 35-mer aptamer, (B) separate split aptamer fragments, and (C) split aptamer [E1 + E2]. The ratio in the sample solution containing estradiol $[(A_{650}/A_{520})_X]$ and the blank solution without estradiol $[(A_{650}/A_{520})_0]$ were recorded to calculate the values of $\Delta A_{650}/A_{520}$ [$\Delta A_{650}/A_{520} = (A_{650}/A_{520})_X - (A_{650}/A_{520})_0$].

the original aptamer optimizes the signaling properties of this assay, including both its dynamic range and sensitivity. When a split aptamer with a high free energy of the secondary structure is used, the assay exhibits high sensitivity and a broad dynamic range. Conversely, when a split aptamer with a low free energy is used, the assay exhibits poor sensitivity. Moreover, the sensors can recognize the target in complex samples with a tunable dynamic range, thus displaying potential for clinical applications. This study reinforces the prospect of using split aptamers under simpler DNA structures in various colorimetric assays.

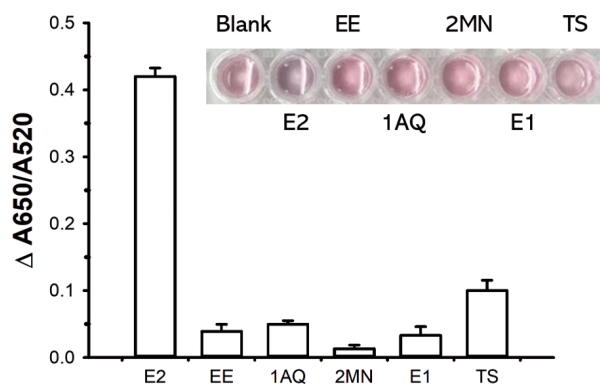


Fig. 4 Selectivity evaluation of the split aptamer-based AuNP colorimetric assay. Photographs of the split aptamer in the AuNP solution after the addition of interference molecules are shown in the inset. Final concentrations of estradiol and other interference molecules were $6.7 \mu M$.

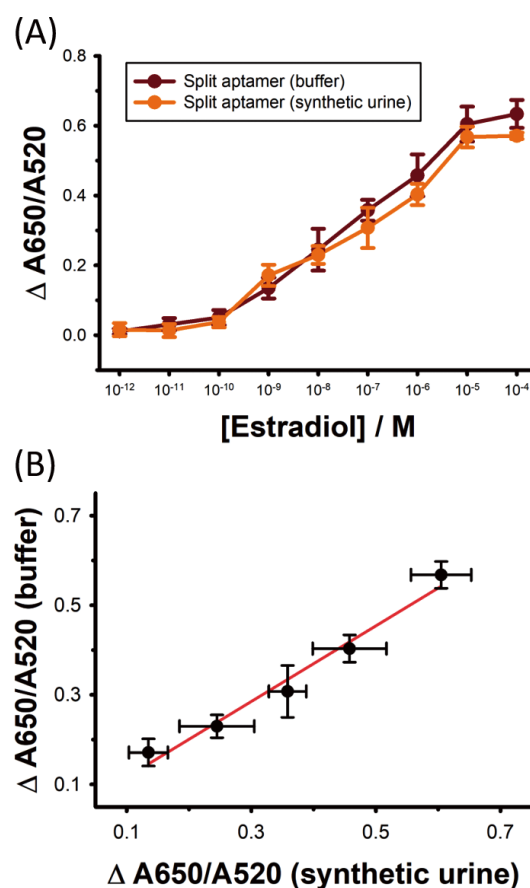


Fig. 5 (A) Colorimetric aptasensor responses toward a range of estradiol concentrations in spiked urine and buffer. (B) Correlation between the results obtained in the urine samples and the buffer samples of estradiol with five concentrations (6.7×10^{-9} , 6.7×10^{-8} , 6.7×10^{-7} , 6.7×10^{-6} , and 6.7×10^{-5} M) ($R^2 = 0.98$).

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Supporting Information

Selectivity evaluation using the original 35-mer aptamer (Fig. S1) and dilution factor without estradiol (Fig. S2) are shown. This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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