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Aptamer-Based Optical Biosensor For Rapid and Sensitive Detection of 17β-Estradiol In Water Samples

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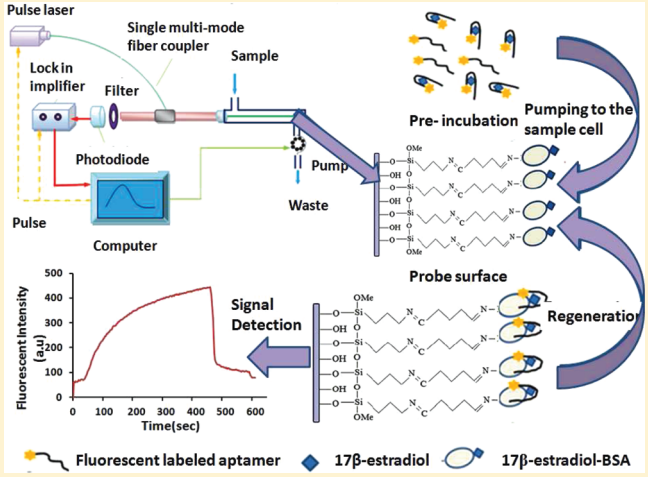
Supporting Information

ABSTRACT: Required routine monitoring of endocrine disrupting compounds (EDCs) in water samples, as posed by EPA Unregulated Contaminant Regulation (UCMR3), demands for cost-effective, reliable and sensitive EDC detection methods. This study reports a reusable evanescent wave aptamer-based biosensor for rapid, sensitive and highly selective detection of 17β-estradiol, an EDC that is frequently detected in environmental water samples. In this system, the capture molecular, β-estradiol 6-(O-carboxy-methyl)oxime-BSA, was covalently immobilized onto the optical fiber sensor surface. With an indirect competitive detection mode, samples containing different concentrations of 17β-estradiol were premixed with a given concentration of fluorescence-labeled DNA aptamer, which highly specifically binds to 17β-estradiol. Then, the sample mixture is pumped to the sensor surface, and a higher concentration of 17β-estradiol leads to less fluorescence-labeled DNA aptamer bound to the sensor surface and thus to lower fluorescence signal. The dose–response curve of 17β-estradiol was established and a detection limit was determined as 2.1 nM (0.6 ng mL^{-1}). The high specificity and selectivity of the sensor were demonstrated by evaluating its response to a number of potentially interfering EDCs. Potential interference of real environmental sample matrix was assessed by spiked samples in several tertiary wastewater effluents. The sensor can be regenerated with a 0.5% SDS solution (pH 1.9) over tens of times without significant deterioration of the sensor performance. This portable sensor system can be potentially applied for on-site real-time inexpensive and easy-to-use monitoring of 17β-estradiol in environmental samples such as effluents or water bodies.



INTRODUCTION

Endocrine disrupting compounds (EDCs) are contaminants of emerging concern due to their harmful effects on endocrine function of human and aquatic organisms.[1] Steroid hormones such as 17β-estrodial, testosterone, and androstenedione is a category of EDCs that has been found frequently in natural water sources and in wastewater effluents in the range of 0.2 to 3 mg L^{-1}.[2] 17β-estradiol, a natural estrogen excreted by humans and domestic animals, has the greatest estrogenic activity and has been detected in various water samples.[3,4] 17β-Estradiol entering into the organism body from outside interferes with the normal physiological processes and creates many deleterious effects.[5] For example, 17β-estradiol may affect the reproductive health of humans and animals through the food chain, particularly leads to an abnormality of growth and function of the male reproductive system, influence the decline of male birth rate and can also induce the rise of incidence of tumor.[2] The EPA Unregulated Contaminant Regulation (UCMR3) has proposed to require monitoring of a number of EDCs, including 17β-estradiol, atrazine, and nonylphenol (NP) in wastewater samples. Therefore, it is of great need to establish sensitive, rapid and automatic methods for the detection of 17β-estradiol within natural systems for protecting environment and public health.[6]

Considerable research interests, therefore, have risen for detecting low level of EDCs, both in advanced instrumental analysis and in biosensor developments.[2] Instrumental analysis methods, such as HPLC or GC/MS, are very sensitive at detecting these toxic chemicals, however, the analytical procedure are rather complicated and therefore labor-intensive and time-consuming.[6] As an alternative, various chemical and biological sensors have been explored for detection of these trace pollutants.[7] Several biosensor systems have been

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developed for the determination of 17β-estradiol using human estrogen-receptor (hER).[7,8] These biosensors can detect estrogenic chemicals including 17β-estradiol with good sensitivity, however, their specificity are compromised due to the nonspecific and wide range of affinity of estrogen-receptor to other xenoendocrines.[9,10] Recently, the immunosensors and nanobiosensors have also been developed for sensitive and rapid detection of 17β-estradiol by utilizing antibodies or functional polymers.[11−14] The specificities of these biosensors were reported to be better than hER-based biosensors due to their structure-specific biorecognition.[15]

This study aimed to develop an optical fiber sensor platform-based biosensor for the detection of 17β-estradiol using a short chain oligonucleotide (aptamer), which was selected by SELEX process and has high affinity and specificity for 17β-estradiol.[16] Aptamers offer a useful alternative to antibodies as sensing molecules and have opened a new era in development of affinity biosensing due to their unique characterizations.[17] In vitro selected aptamers could be produced for any targets such as proteins, peptides, amino acids, nucleotides, drugs, carbohydrates, and other small organic and inorganic compounds,[18,19] and could be chemically synthesized without the complicated and expensive purification steps for antibody production. In addition, aptamers are more stable than antibodies and thus are more resistant to denaturation and degradation.[19] Herein, using a compact, inexpensive, and easy-to-use evanescent wave fiber-optic biosensor platform, we report a novel biosensor for rapid, highly specific and sensitive detection of 17β-estradiol based on fluorescence-labeled aptamer for specifically binding 17β-estradiol. The biosensor's sensing time, sensitivity, specificity, resistance to background interference, and reusability were evaluated.

MATERIALS AND METHODS

Chemicals and DNA Aptamer Oligonucleotides. 17β-Estradiol was purchased from MP Biomedicals (USA), 17β-estradiol 6-(O-carboxymethyl) oxime bovine serum albumin (17β-estradiol -BSA), 3-aminopropyltriethoxysilane(APTS), and glutaraldehyde (GA) were purchased from Sigma-Aldrich (St. Louis, MO). A single-stranded DNA aptamer against 17β-estradiol has been isolated by SELEX process from a random ssDNA library by Y.S. Kim et al.[16] Fluorescent dye labeled 17β-estradiol-aptamer; 5′-(Cy5.5)-GCT-TCC-AGC-TTA-TTG-AAT-TAC-ACG-CAG-AGG-GTA-GCG-GCT-CTG-CGC-ATT-CAA-TTG-CTG-CGC-GCT-GAA-GCG-CGG-AAG-C-3′ and nonspecific DNA sequence (used for control) 5′-Cy5.5-TCCCGAGA-3′ were purchased from Eurofins MWG Operon (U.S.).

Buffer solution of 100 mM Tris−HCl was used for all experiments, which contained 200 mM NaCl, 25 mM KCl, 10 mM $MgCl_2$ and 5% ethanol and had a pH of 8.0. Both aptamer and nonspecific DNA oligonucleotides were dissolved in 100 mM Tris-HCL and kept frozen at −20 °C for storage. 17β-Estradiol was dissolved with methanol as the stock solutions and diluted using the buffer solution for analysis. Other EDCs and chemicals include Carbaryl, 4-*n*-nonylphenol (4NNP) (both from Riedel-de Haen, U.S.), Estriol (from MP Biomedicals, U.S.), Lincomycin Hydrochloride, 1-ethyl-3-[3-(dimethylamino) propyl]carbodiimide (EDPC) and Mitomycin C, (all from Thermo Fisher Scientific Inc., U.S.).

Instrumentation: Evanescent Wave All-Fiber Bisensing Platform. The portable evanescent wave all-fiber portable biosensing platform was as previously described.[20] Briefly, the laser beam from a 635-nm pulse diode laser with pigtail was directly launched into a single-mode fiber of a single multimode fiber coupler. The laser light then entered the multimode fiber with a diameter of 600 μm and numerical aperture of 0.22 from the single-mode fiber. The excitation light from the laser, through the fiber connector, was coupled to a fiber probe. The incident light propagated along the length of the probe via total internal reflection. The evanescent wave generated at the surface of the probe then interacted with the surface-bound fluorescently labeled analyte complexes and caused excitation of the fluorophores. The collected fluorescence was filtered by means of a bandpass filter and detected by photodiodes through a lock-in detection. The probe was embedded in a glass flow cell with a flow channel having a nominal dimension of 60 mm in length and 2 mm in diameter. All reagents were delivered by a flow delivery system operated with a peristaltic pump. The controls of fluid delivery system, data acquisition and processing were automatically performed by the built-in computer.

Immobilization of 17β-Estradiol-BSA onto Optical Fiber Sensor Surface. Details of the fabrication and preparation of the combination tapered fiber optical sensor were previously described.[21] Prior to surface modification, the sensor surface was cleaned with a piranha solution (H_2SO_4/H_2O_2, 3:1 [v/v]) for 30 min, and rinsed with ultrapure water and dried in an oven at 105 °C. The immobilization process of 17β-estradiol-BSA onto the surface of optical fiber sensor is illustrated in the Figure 1. The optical fiber sensor was first

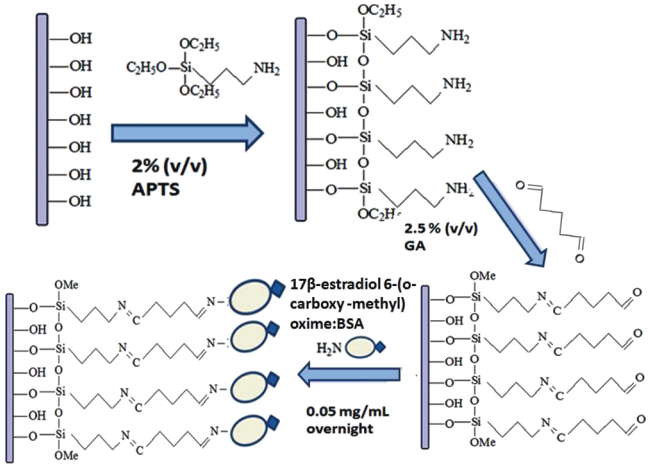

Figure 1. Schematics of the process employed for immobilization of molecular β-estradiol-BSA onto the sensor surface using glutaraldehyde covalent coupling approach.

aminated by immersion in a 2% (v/v) APTS acetone solution for 60 min at room temperature, followed by an acetone washing (three times) and ultrapure water washing, and dried in an oven for 30 min at 110 °C. To immobilize 17β-estradiol-BSA onto the surface of amino-silanized sensor, the sensor were at first immersed in 2.5% (v/v) GA solution for 1 h at 37 °C, washed thoroughly with ultrapure water, then immersed in 0.05 mg mL^{-1} 17β-estradiol-BSA in PBS (pH 7.4) solution for overnight at 4 °C. Before each analysis, the sensor was soaked in 2 -mg mL^{-1} BSA for 1 h at room temperature to block the remaining aldehyde sites (more details are described in sensor optimization results).[22]

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Sensing Mechanism. Figures 2 and 3 illustrate the sensing mechanism of the evanescent wave aptamer-based biosensor for

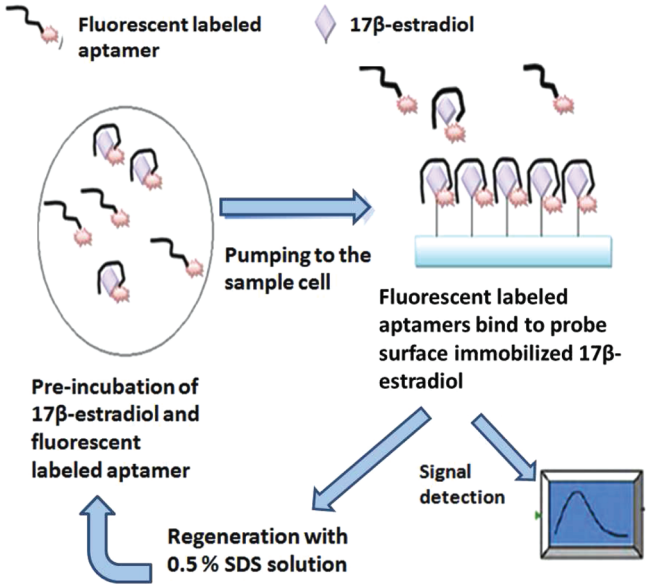

Figure 2. Schematic representation of sensing mechanism of 17β-estradiol detection with the aptamer-based optical biosensor.

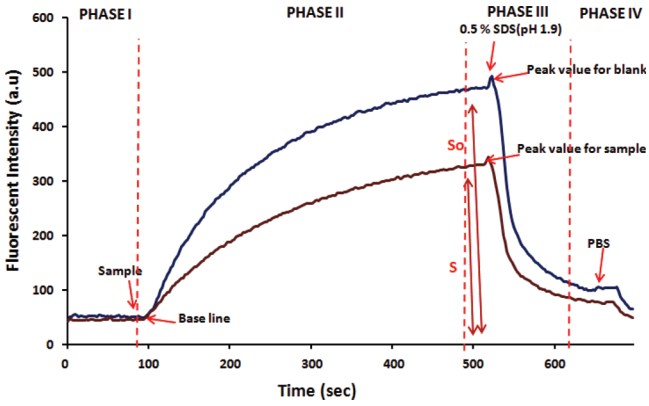

Figure 3. Exemplary signal profiles for 17β-estradiol detection with the aptamer-based optical biosensor.

detection of 17β-estradiol and the exemplary signal profile for 17β-estradiol detection, respectively. The sensing procedure started with BSA injection to the sample cell to avoid the nonspecific binding to the sensor surface. BSA was pumped into a flow cell at a rate of 300 μL/min for 30s. Samples containing various concentrations of 17β-estradiol were mixed with a fixed amount of fluorescence-labeled aptamer DNA for a certain time. Portion of the aptamer specifically bound with 17β-estradiol, and the concentration of free aptamer was inversely proportional to that of 17β-estradiol in samples (Phase I in the Figure 3). Then, the mixture was pumped through the optical fiber sensor surface, and the remaining free aptamer bound to the 17β-estradiol immobilized on the sensor surface, giving a fluorescence signal which is target 17β-estradiol concentration-dependent (Phase II in the Figure 3). The samples were pumped through the sensor for 1 min at a rate of 300 μL/min, then the sample continued to react with 17β-estradiol immobilized on the sensor surface for other 4 min and the fluorescence signal was recorded real-time. To reuse the sensor, the sensing surface was regenerated with a 0.5% SDS solution (pH 1.9) for 90 s (Phase III in the Figure 3) and washed with a PBS solution (Phase IV in the Figure 3).

Figure 3 shows the exemplary real time signals for one analysis cycle. The signal for each assay, which is used for dose response relationships later, is calculated as follow:

S_0 = (Fluorescent intensity at the peak value − Fluorescent intensity at the baseline) of blank

S = (Fluorescent intensity at the peak value − Fluorescent intensity at the baseline) of sample

ΔS = Signal = $S_0 - S$ (a.u., fluorescence intensity)

Optimization of the Sensing Conditions. Studies were performed for various sensing steps to identify the optimal sensing conditions, including incubation time of sample, concentration of aptamer in prereaction mixture and nonspecific sites blocking of sensing surface with BSA. To identify the effect of the incubation time of 17β-estradiol and fluorescence labeled aptamer on the sensing performances, the different incubation time of 5, 10, and 20 min was conducted and compared.

To evaluate the effect of BSA to block the nonspecific adsorption sites on the sensor surface, we performed and compared two approaches. In the first approach, before detecting samples, we pump BSA through the sensor cell to block the nonspecific adsorption for 30 s and wash the sensor surface via PBS buffer solution (30 s) to remove the residual of BSA. In the second design, we premixed BSA and fluorescence-labeled aptamer before sampling. To obtain the optimal BSA concentration, 0.5, 1, and 2 mg mL^{-1}BSA was tested, respectively.

Assessment of Sensor Specificity. To determine the specificity of the aptamer used, several EDCs such as Estriol, Carbaryl, 4-*n*-nonylphenol (4NNP), and a few other chemicals, including Lincomycin Hydrochloride, 1-ethyl-3-[3-(dimethylamino) propyl] carbodiimide (EDPC) and Mitomycin C, were tested.

Analysis of Spiked Wastewater Treatment Effluent Samples. To evaluate the potential matrix effect of real environmental water sample on the sensor performance, we analyzed spiked samples that contained different concentrations of 17β-estradiol (5 nM, 10 nM and 25 nM) in four different wastewater effluents from three wastewater treatment plants in the U.S. The wastewater effluent samples were filtered through 0.22 μm filters to remove all particulates before they were spiked with 17β-estradiol. Two duplicate experiments were performed for all samples. Similar analytical procedures were followed as described above.

RESULTS AND DISCUSSION

Immobilization of 17β-Estradiol-BSA onto Sensor Surface. Covalent immobilization of 17β-estradiol-BSA on an aminated fiber optic sensing surface was achieved using a glutaraldehyde coupling strategy (Figure 1). To evaluate the immobilization results and to confirm that the observed fluorescence signal was from binding between 17β-estradiol immobilized onto sensor surface and fluorescence-labeled aptamer, we conducted several control experiments whose results are shown in Figure 4. For analysis conducted without BSA blocking, as shown in Figure 4, nonspecific binding occurred with nsDNA. However, after pumping the BSA to the sample cell, 100 nM fluorescently labeled nonspecific DNA (nsDNA, represents other DNA rather than the specific

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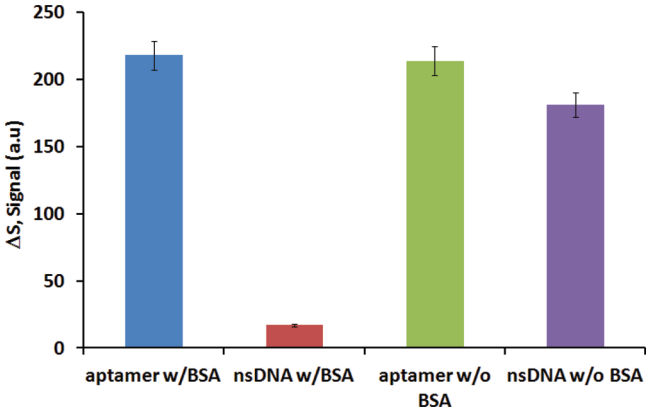

Figure 4. Assessment of the immobilization effectiveness and specific binding between 17β-estradiol (immobilized onto sensor surface) and fluorescence-labeled aptamer. Here w/BSA or w/o BSA represents the experiment with or without BSA injection to block the nonspecific binding sites, nsDNA represents the experiments with nonspecific DNA (nsDNA) sequence as control instead of aptamer that binds specifically to 17β-estradiol.

aptamer), was delivered over the sensing surface; no fluorescent signal was observed, thereby indicating no nonspecific adsorption of DNA onto surface. On the basis of the results shown in Figure 4, another important conclusion, beside that BSA was effective for blocking nonspecific binding, was that BSA treatment did not interfere with the interaction between the aptamer and the immobilized 17β-estradiol-BSA conjugates. When 100 nM Cy5.5 dye-labeled aptamer was introduced, however, a strong fluorescent signal was detected; indicating binding of the aptamer to the sensor surface and a signal-to-noise ratio (the ratio of the maximum fluorescent signal to the baseline) of over 9.8 was obtained (Figure 3). As the sample with mixture of aptamer and 17β-estradiol is introduced, the signal also increased, but to a less extent compared to those with no 17β-estradiol, indicating that the target indeed bounded to some of the aptamer, led to less fluorescence signal (Figure 3). The decrease of the signal with target present compared to that with only aptamer in buffer blank is calculated as the final sensor signal correspond to a certain target concentration as previously described. These results demonstrated the ability of the sensor for detecting the binding between specific aptamer and 17β-estradiol.

Optimization of the Sensing Conditions. Studies were performed for various sensing steps to identify the optimal sensing conditions, including incubation time of sample and concentration of aptamer in prereaction mixture and nonspecific sites blocking of sensing surface with BSA. To optimize the aptamer concentration, 10, 25, 50, 100, and 150 nM concentration of fluorescent-labeled aptamer DNA were tried for this experiment. The signals for different concentration of fluorescent labeled DNA are demonstrated in Figure S1 of the Supporting Information, SI, and results showed that 100 nM DNA is near the initial signal saturation level for the evanescent wave aptamer-based biosensor. Therefore, 100 nM fluorescent labeled DNA was applied in all the following experiments.

Several incubation times (5, 10, and 20 min) for premixing of 17β-estradiol and fluorescence labeled 17β-estradiol-aptamer were considered (Figure S2 of the SI). We observed that prolonged incubation time of the 17β-estradiol with aptamer led to slightly increase in the sensor signal, indicating less aptamer bound with the 17β-estradiol. The reason for this is unclear, which might be related to the stability of the aptamer-17β-estradiol complex and/or the potential redissolution of 17β-estradiol from buffer solution over time. Nevertheless, results with 5 and 10 min incubation time were fairly consistent; therefore, we chose to use 10 min incubation time for all the subsequent analysis.

To examine the effective approach for blocking the nonspecific binding sites, we tried two methods. We tried to pump the BSA solution through the sensor cell before sampling or mixing the BSA with 17β-estradiol and Cy5.5 dye labeled-aptamer sample before pumping. Figure S3 of the SI shows that premixing of BSA and Cy5.5 dye labeled 17β-estradiol-aptamer sample is not suitable for this experiment. One possible explanation is that when we mix BSA, aptamer and sample together, BSA can bind not only to the sites on the probe surface but also link to 17β-estradiol. However, pretreating the sensor surface with BSA then followed by washing was shown to be effective for blocking the unspecific sites before sampling. We then further optimized the BSA concentration and for this purpose, we tried and compared results with three different concentrations (0.5, 1, and 2 mg mL^{-1}) of BSA. The results showed that there is not any distinctive difference in the results with these different BSA concentrations (data not shown). Therefore, we applied 1 mg/L BSA for all subsequent experiments.

Dose–Response Measurements of the Sensor. Figure 5 shows the exemplary temporal fluorescence intensity during a typical test cycle for 17β-estradiol detection using the optical sensor scheme developed herein, including the BSA treatment for blocking nonspecific sites, introduction of a mixture of the fluorescence-labeled aptamer and 17β-estradiol, the binding between the aptamer and the 17β-estradiol immobilized on the sensor probe, and sensor regeneration. The introduction of 17β-estradiol at different concentrations to the sensing interface induced proportional decrease in the fluorescence signal.

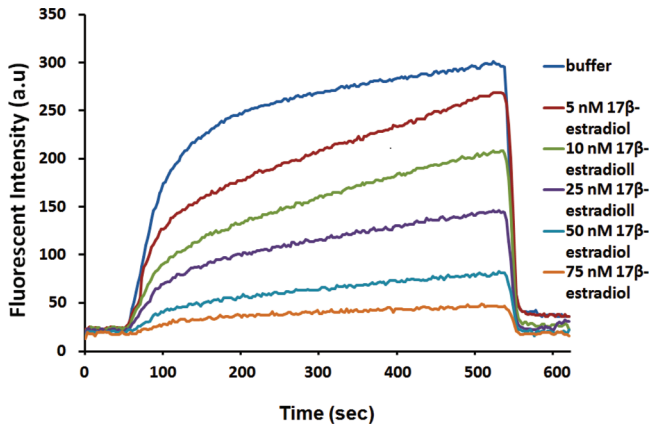

Figure 5. Exemplary sensor response curves and the signals obtained with various concentration of 17β-estradiol.

Figure 6 shows the calibration curve for 17β-estradiol, which was normalized by expressing the signal decreases of each standard point as the ratio to that of the blank sample containing no 17β-estradiol. The signal intensities were fitted to a 4-parameter logistic equation as follows:

$$y = \frac{A_1 - A_2}{1 + ([Ac]/[Ac_0])^p} + A_2 \quad (1)$$

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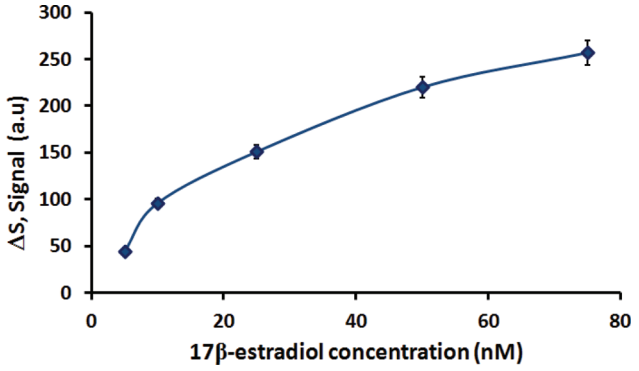

Figure 6. Logarithmic calibration plot for determination of 17β-estradiol using aptamer-based fiber optic biosensor system.

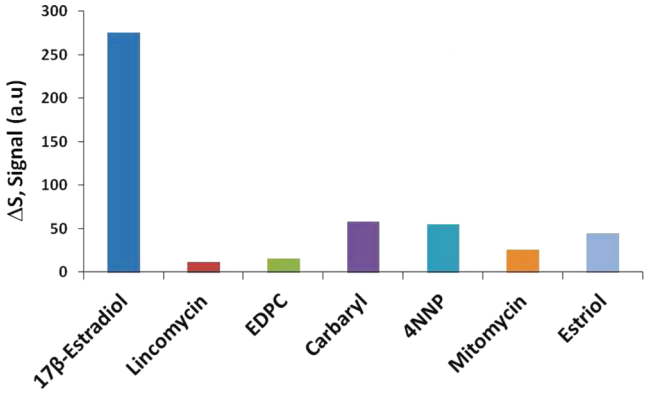

Figure 7. Comparison of sensor signals with 17β-estradial and other EDCs and chemicals.

where, [Ac] is the analyte concentration; A_1, A_2 are the upper and lower asymptote (background signal) to the titration curve; [Ac_0] is the analyte concentration at inflection; and p is the slope at the inflection point.[20] The error bars in the figure correspond to the standard deviations of the data points in triplicate experiments, with coefficient of variance (CV) of all the data points being within 5%.

On the basis of the dose−response curve and the signal-to-noise ratio of 9.8, the detection limit was determined as approximately 2.1 nM (0.6 ng mL^{-1}) based on average standard deviation of measurements (σ) and slope of dose−response (S) fitting curve as $3\sigma/S$.[27] The detection limit we obtained initially is comparable to those reported in the electrochemical detection of 17β-estradiol using DNA aptamer immobilized gold electrode chip[16] which has the detection limit 0.3 $ngmL^{-1}$ and the disposable amperometric immunosensor for the detection of 17β-estradiol using screen-printed electrodes[6] which has the detection limit 0.5 $ngmL^{-1}$. The detection limit is also comparable to chemical analysis with standard HPLC that has a reported detection limit of 1.15 nM for 17β-estradiol.[26] Considering the range of 17β-estradiol concentrations (<0.2−3 ng mL^{-1}) detected in natural waters.,[23] our novel sensor can be applied for detection of 17β-estradiol in natural or wastewater samples. In addition, compared to the sensors mentioned above, the sensor developed here is simpler and faster (less than 10 min, including measurement and regeneration). And the portable platform also allows for potential on-site or real time measurements.

Specificity of the Sensor. Specificity of the sensor was assessed by analysis of other EDCs such as Carbaryl, 4-*n*-nonylphenol (4NNP), estriol, as well as several other chemicals including Lincomycin Hydrochloride, 1-ethyl-3-[3-(dimethylamino) propyl]carbodiimide (EDPC) and Mitomycin C, all at 20 ng- mL^{-1} (Figure 7). The results showed that the developed biosensor system have high specificity toward 17β-estradiol, with much lower signal for all other EDCs and chemicals tested.

Regeneration and Sensor Stability. In the assays herein described, 17β-estradiol-BSA conjugates were immobilized to the probe surface using glutaraldehyde covalent coupling approach as previously described. The regeneration of sensor surface can be achieved using regeneration solution (a 0.5% SDS solution, pH 1.9) without damage of its physical−chemical properties, which is essential for the repeated use of the sensor probe. The complete removal of noncovalently bound fluorescence-labeled aptamer for 17β-estradiol-BSA conjugates was achieved using regeneration solution at 300 μL min^{-1} (Figure S4 of the SI). Using this regeneration agent, the sensor surface was reused for more than hundred of measurements without any significant loss of performance during the 4-months testing period of this study.

The main advantage of biosensors over other detection methods (e.g., ELISA formats) is the better regeneration and binding properties of the sensing surface, which is critical for the successful reuse of the same sensor surface and the accuracy of detection results.[24,25] The developed biosensor platform here has the same successful reusable property. When the probe was stored in a refrigerator at 4 °C and measured intermittently (every 5 days), after 90 days of storage, the optical biosensor system retained about 90% of its original response.[20]

Wastewater Samples Analysis. To study the influence of matrix effect on the fiber optic biosensor response, several samples spiked with 17β-estradiol, with concentrations of 5, 10, and 25 nM, were tested by spiking the filtered wastewater effluent samples. The results were summarized in Table 1. The recovery of all measured samples was between 95 and 105%, and the parallel tests showed that the relativity coefficient was more than 0.99 (n = 2). These results indicated that the possible interference from the different background composition of water samples on aptamer based fiber optic biosensor analysis was negligible. The developed biosensor system can be successfully applied to 17β-estradiol analysis in real environmental water samples.

In conclusion, we have developed an aptamer-based evanescent wave optical biosensor for rapid and selective 17β-estradiol using a portable and easy-to-use all-fiber biosensing platform. The sensing process can be completed in less than 10 min, with a detection limit of 2.1 nM. The robustness of the 17β-estradiol-BSA conjugate surface for biorecognition molecule immobilization and the effective surface regeneration procedures allow many assay cycles without any significant loss of its performance. The performance of the biosensor evaluated in spiked wastewater samples showed good recovery, precision and accuracy, indicating that it was not susceptible to water matrix interferences even without the need of complicate sample pretreatments. All of these results illustrated that the biosensor developed here could be readily extended toward the on-site monitoring of the other trace small molecular pollutants in environmental matrices with the employment of different probes modified by other analyte conjugates and fluorescence-labeled aptamers.

Table 1. Detection Results of 17β-Estradiol-Spiked Wastewater Effluent Samples

Sample source	Treatment process and effluent	17β-Estradiol added to the samples (nM)	17β-Estradiol detected by sensor (nM)	Coefficient of variation (CV) %	Recovery %
Plant A treatment train 1	Membrane Bioreactor effluent	5	4.79	1.3	95.7
		10	9.74	2.4	97.4
		25	23.52	3.4	94.1
Plant B	Tertiary multi-stage filtration effluent	5	5.24	2.5	104.8
		10	10.28	3.6	102.8
		25	25.85	3.9	103.4
Plant A treatment train 2	Continuously up-flow media filtration effluent	5	5.26	3.9	105.3
		10	9.92	3.2	99.2
		25	26.13	2.5	104.5
Plant C	Advanced tertiary effluent	5	4.71	3.8	94.2
		10	9.42	2.9	94.2
		25	23.95	3.5	95.8

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed descriptions of the sensing mechanism such as fluorescent labeled aptamer concentration optimization (Figure S1), premixing time optimization (Figure S2), blocking the nonspecific binding sites in the probe surface with BSA (Figure S3), and sensor surface regeneration (Figure S4) are available in the supporting materials. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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