Environmental Science & Technology

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Screening of Endocrine Disrupting Potential of Surface Waters via an Affinity-Based Biosensor in a Rural Community in the Yellow River Basin, China

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Cite This: https://doi.org/10.1021/acs.est.2c01323

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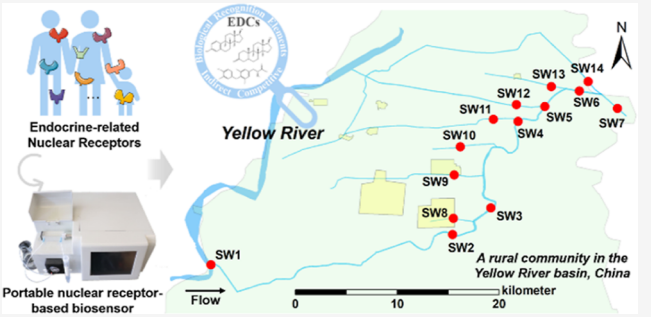
ABSTRACT: Overcoming the limitations of traditional analytical methods and developing technologies to continuously monitor environments and produce a comprehensive picture of potential endocrine-disrupting chemicals (EDCs) has been an ongoing challenge. Herein, we developed a portable nuclear receptor (NR)-based biosensor within 90 min to perform highly sensitive analyses of a broad range of EDCs in environmental water samples. Based on the specific binding of the fluorescence−labeled NRs with their ligands, the receptors were attached to the EDC−functionalized fiber surface by competing with EDCs in the samples. The biosensor emitted fluorescence due to the evanescent wave excitation, thereby resulting in a turn-off sensing mode. The biosensor showed a detection limit of 5 ng/L E_2-binding activity equivalent (E_2-BAE) and 93 ng/L T_3-BAE. As a case study, the biosensor was used to map the estrogenic binding activities of surface waters obtained from a rural community in the Yellow River basin in China. When the results obtained were compared with those from the traditional yeast two-hybrid bioassay, a high correlation was observed. It is anticipated that the good universality and versatility exhibited by this biosensor for various EDCs, which is achieved by using different NRs, will significantly promote the continuous assessment of global EDCs.



KEYWORDS: *endocrine-disrupting chemicals, indirect competition bioassay, nuclear receptors, evanescent wave biosensor, affinity-based, rural community*

INTRODUCTION

It has been widely reported that chronic, low-level exposure to endocrine-disrupting chemicals (EDCs) can inadvertently cause adverse health effects.[1] Because of their widespread presence in the environment, EDCs have been listed as an environmental issue that necessitates global cooperation by the United Nations Environment Program.[2] Therefore, it is crucial to identify and quantify the potential EDCs in complex environments to perform environmental monitoring and risk assessments. However, EDCs show great phyletic complexity and broad structural diversity. Some conventional instrumental analytical techniques, such as high-performance liquid chromatography (HPLC), gas chromatography−mass spectrometry (GC−MS), and liquid chromatography tandem−mass spectrometry (LC−MS/MS), cannot screen all broad ranges of EDCs, especially compounds that have not been previously recognized.[3] Therefore, a facile, efficient, and reliable method to quantify and assess total EDCs is in high demand, but despite global efforts, developing such as method remains a significant challenge.

It is well-accepted that the binding or modulation of nuclear hormone receptors is a major mode of action of EDCs.[2,4] Nuclear receptors (NRs) are a class of ligand-dependent transcription factors that are widely distributed in organisms, and NRs are divided into three categories, including steroidal nuclear receptors (SNRs), non-steroidal nuclear receptors (non-SNRs), and orphan nuclear receptors (ONRs).[5] Most NRs are classified as ONRs due to the lack of well-characterized ligands.[6] In contrast, a large number of ligands are known for virtually all SNRs and non-SNRs, and receptor−ligand affinity interactions generally occur in the C-terminal ligand binding domain (LBD).[7] To date, approximately 12 endocrine-related classic NRs with different structures and classifications (Figure S1) have been reported. The specific

Received: February 22, 2022
Revised: September 8, 2022
Accepted: September 9, 2022

ligands trigger some common endocrine-disrupting effects, such as estrogenic-disrupting effect, androgenic-disrupting effect, thyroid hormone-disrupting effect, and progesterone-disrupting effect.[4] Based on the above specific interactions between NRs and their ligands, cell-dependent bioassays, such as CALUX, HAhLP, MVLN, E-Screen, and yeast two-hybrid bioassay, have been developed and widely used to screen broad-ranging EDCs.[8−10] However, the majority of these bioassays require multi-day operations, specialized operators, or sophisticated equipment. Moreover, these methods involve possible cytotoxic effects and transmembrane transport under complex and unpredictable environmental conditions.[11] To overcome these deficiencies, cell-free bioassays based on the specific interaction of receptors and their ligands have been widely exploited,[12,13] although the denaturation of the hormone receptor, which is less protected when separated from the cell, was challenging to overcome.

Evanescent wave fluorescent biosensors, which are based on the total internal reflection phenomenon and near-surface-captured fluorescent signals, yield excellent sensitivity and selectivity.[14] A series of regenerable evanescent wave fluorescent biosensors, in which steady advancements have been achieved to accurately detect antibiotics, toxins, heavy metal ions, pesticides, and so on, have been developed by using aptamers, antibodies, and functional nucleic acids.[15−19] These previous studies greatly promoted the application of evanescent wave fluorescent biosensors in monitoring various environmental pollutants that are widely concerning. However, biological recognition elements that are frequently used, including aptamers, antibodies, and functional nucleic acids, usually can only quantify a single target and fail to detect a broad range of compounds, especially unrecognized compounds.[20−22] Inspired by the endocrine-disrupting actions of EDCs, NRs have been utilized as biological recognition elements in biosensing platforms. The biosensor developed thereby can screen and quantify the same class of EDCs that exhibit a specific endocrine-disrupting effect.[23−25] Although good performance was achieved, these studies exhibited a few disadvantages; for example, the sensing strategies were convoluted and contained a multi-step recognition process, resulting in weakened assay sensitivity and a time-consuming detection process (approximately 8 h). Therefore, establishing a rational design of NR-based biosensors to map a comprehensive picture of potential estrogenic-disrupting chemicals remains a highly desirable yet challenging goal.

To address the abovementioned research gap, we developed a NRs-based biosensor to screen potential EDCs in environmental water samples. On account of the concise indirect competitive bioassay strategy, the affinity-based biosensor easily achieves the screening and quantification of EDCs with a ng/L range sensitivity, fulfilling regulatory demands. Synthetic chemicals with potential endocrine-disrupting effects, such as pesticides, fertilizers, and pharmaceutical and personal care products, are widely utilized for agriculture, stock farming, and livelihoods in rural communities.[26] Therefore, we mapped the estrogenic binding activities of various surface waters obtained from a rural community in the Yellow River basin in China using the developed biosensor. The automated technology to monitor EDCs with high sensitivity is anticipated to significantly promote the continuous assessment of endocrine disruptors in global environments.

MATERIALS AND METHODS

The materials, reagents, and buffers used in this work are described in the Supporting Information (Section S1).

Fluorescent Labeling the hERα−LBD and Activity Verification. The construction and characterization of the recombinant human estrogen receptor α−LBD (hERα−LBD) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis is described in the Supporting Information (section S3 and Figure S2). The fluorescent labeling procedure of the hERα−LBD was performed according to the method described in our previous work with slight modifications, and details can be found in the Supporting Information (section S4).[23]

We investigated the impact of the fluorescent labeling process on the binding activity of the hERα−LBD by HPLC with the following setup in five independent experiments. First, 0.1 mL of 20 μM hERα−LBD or 0.1 mL of 20 μM Cy5.5−hERα−LBD solution was incubated with 0.1 mL of Ni-NTA-agarose resin for 2 h to form the resin−receptor complexes due to the binding between the His tag of the hERα−LBD and Ni-charged resin. After centrifugation at 5000*g* for 3 min, the supernatant was removed. Next, 0.1 mL of E_2 solution with different concentrations (2.72×10^6, 4.08×10^6, 5.44×10^6, 8.16×10^6, and 1.088×10^7 ng/L, that is, 10, 15, 20, 30, and 40 μM) was added to the precipitates and incubated for 90 min at room temperature for different reaction molar ratios. After being washed three times, the resin−receptor complexes were incubated with 0.1 mL of methanol solution to denature the receptor and release the bound E_2. Finally, the eluted E_2 was measured by HPLC to calculate the retained ratio of E_2 and to assess the binding activity of the hERα−LBD before and after fluorescent labeling.

Preparation of Estradiol Derivative−Bovine Serum Albumin Conjugates. To ensure that the high affinity and binding performance of Cy5.5−hERα−LBD and E_2-BSA conjugates was not affected by the steric hindrance of bovine serum albumin (BSA), estradiol derivatives with various carbon linking arms E_2−*X*C (*X* = 4, 8, and 10) were conjugated with BSA (Figure S3). Briefly, 10 mM E_2−*X*C, 40 mM EDC, and 60 mM NHS were gently mixed in DMF solution for 4 h at room temperature in the dark. Afterward, the above suspension was incubated with 5 mg/mL BSA (the molar ratio of E_2−*X*C and BSA was 20:1) in coupling buffer for 2 h to construct E_2−*X*C−BSA conjugates (*X* = 4, 8, and 10). To purify the conjugates, the mixture was dialyzed against the coupling buffer for 12 h at 4 °C. Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) was used to characterize the E_2−*X*C−BSA conjugates. In addition, the commercial E_2−BSA conjugate β-estradiol 6-(*o*-carboxymethyl)oxime/BSA was used as a reference.

Preparation of the Reusable Optical Fiber. To ensure that the optical fiber or transducer element exhibited a high reusability, the E_2−*X*C−BSA conjugates were immobilized on the fiber surface by the formation of covalent bonds. As shown in Figure S4, the optical fiber was etched to a tapered structure with hydrofluoric acid before further chemical modifications were performed. Next, the tapered optical fiber was immersed in piranha solution (v/v H_2SO_4: 30% H_2O_2 = 3:1) at 70 °C for 1 h to introduce hydroxyl groups. After thoroughly rinsing with DI water, the fiber was placed in a 2% MTS toluene solution for 2 h to acquire thiol groups, and then, the bifunctional cross-linker GMBS was coupled by 1 h incubation at room

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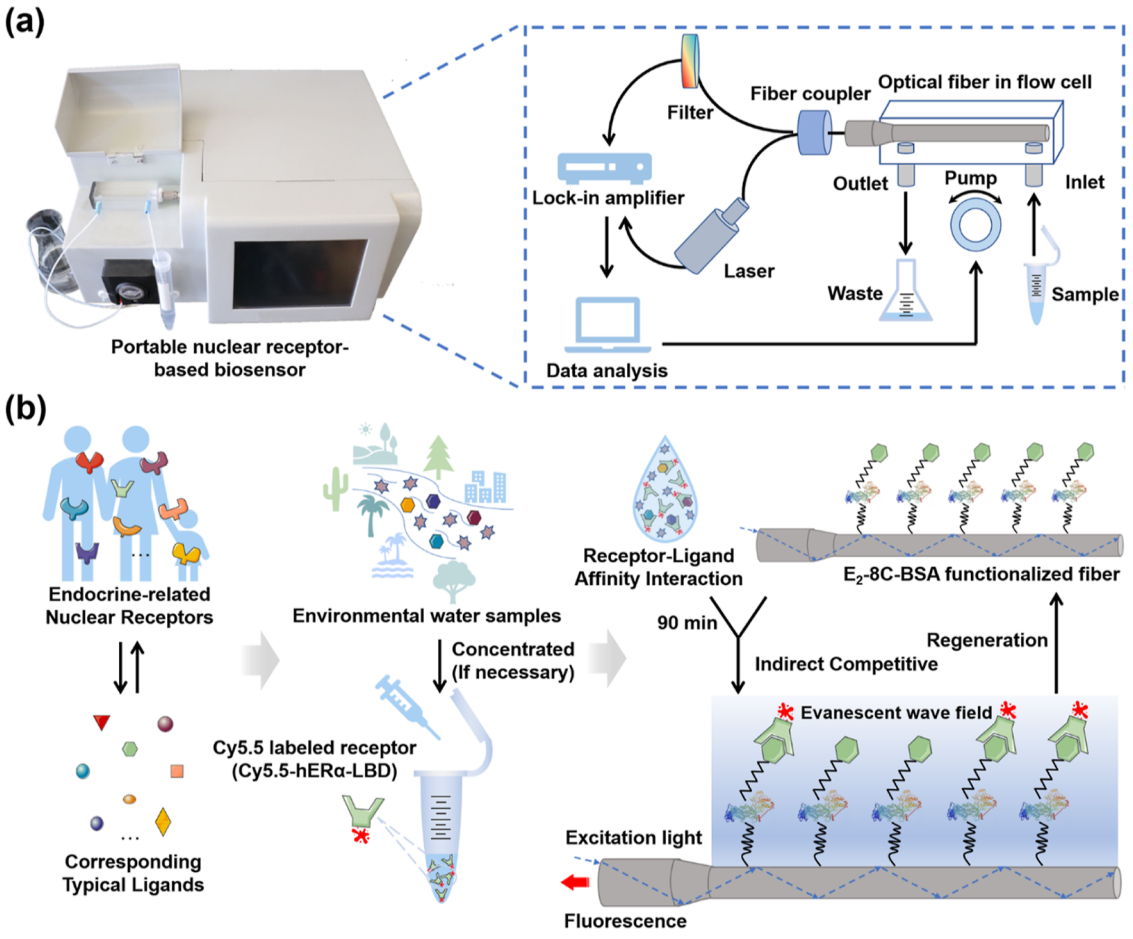

Figure 1. Portable NR-based biosensor for screening of endocrine disrupting potential of environmental water samples. (a) Instrument photograph and structural scheme of the NR-based biosensor and (b) scheme of the sensing mechanism for screening of endocrine disruptors by using the estrogenic disruptors as a typical representative.

temperature. Finally, the fiber was incubated with 0.05 mg/mL E_2−*X*C−BSA conjugates at 4 °C overnight for the covalent coupling reaction between the ester moiety of GMBS and the amino groups of BSA. Before being used, the E_2−*X*C−BSA−functionalized optical fiber was incubated in isoelectric BSA blocking buffer at 4 °C overnight to block the non-specific binding sites.[27] For comparison, the BSA−functionalized optical fiber was prepared using the same method to assess possible non-specific signals.

Quantification of Estrogenic Binding Activity. Under the optimized experimental conditions, the procedures for the quantification of environmental estrogenic-disrupting chemicals were as follows: first, 1 mL of an E_2 standard solution or environmental sample, which was diluted with binding buffer with an optimized dilution ratio of 2:1−3:1, was added into 1 mL of 5 μg/mL Cy5.5−hERα−LBD and incubated for 90 min at room temperature to bind the receptor and ligand. Next, the obtained mixture solution flowed on the E_2−8C−BSA−functionalized optical fiber and was maintained for 5 min to undergo the indirect competitive binding process. Finally, the fluorescent signal of the bound Cy5.5 molecules on the optical fiber surface was detected by the induced fluorescence emission of the evanescent field. Subsequently, the Cy5.5−hERα−LBD conjugate was eluted using 5 mL of washing buffer for 5 min to regenerate the optical fiber sensing element. In the end, the binding buffer was injected on the fiber surface for 5 min, providing a suitable binding environment for the new test cycle. After the calibration curve was established, the environmental estrogenic-disrupting effect was characterized by the E_2-binding activity equivalent (E_2-BAE).

Mapping Estrogenic-disrupting Potential of Surface Waters in a Rural Community in the Yellow River Basin in China. Surface waters were collected from 14 sampling sites in a rural community in the Yellow River basin in China on 13th October 2021. All samples were collected by using a 1 L organic glass hydrophore, stored in cleaned glass bottles, and then transported on wet ice, stored at 4 °C in the dark and extracted within 48 h to be further analyzed using the biosensor and yeast two-hybrid bioassay. To accurately compare the two methods, the samples were pretreated in the same way. First, 500 mL of each water sample was filtered with glass fiber filters (0.7 μm, Whatman GF/F 47 mm, UK) and then enriched by solid-phase extraction with an Oasis HLB column (6 cm^3, 200 mg, Waters, USA). Afterward, the HLB columns were rinsed with DI water, dried under nitrogen, and eluted with methanol. The obtained eluates were concentrated to 1 mL under N_2 and were filtered with nylon membrane filters (0.45 μm, Whatman, UK). The extracts were stored at −20 °C. The obtained 500× water samples were used for the yeast two-hybrid bioassay, and the detailed procedures of the yeast two-hybrid bioassay of estrogenic-disrupting chemicals. EDCs are described in the Supporting Information (section S7). Then, 100 μL of 500× water samples was diluted by

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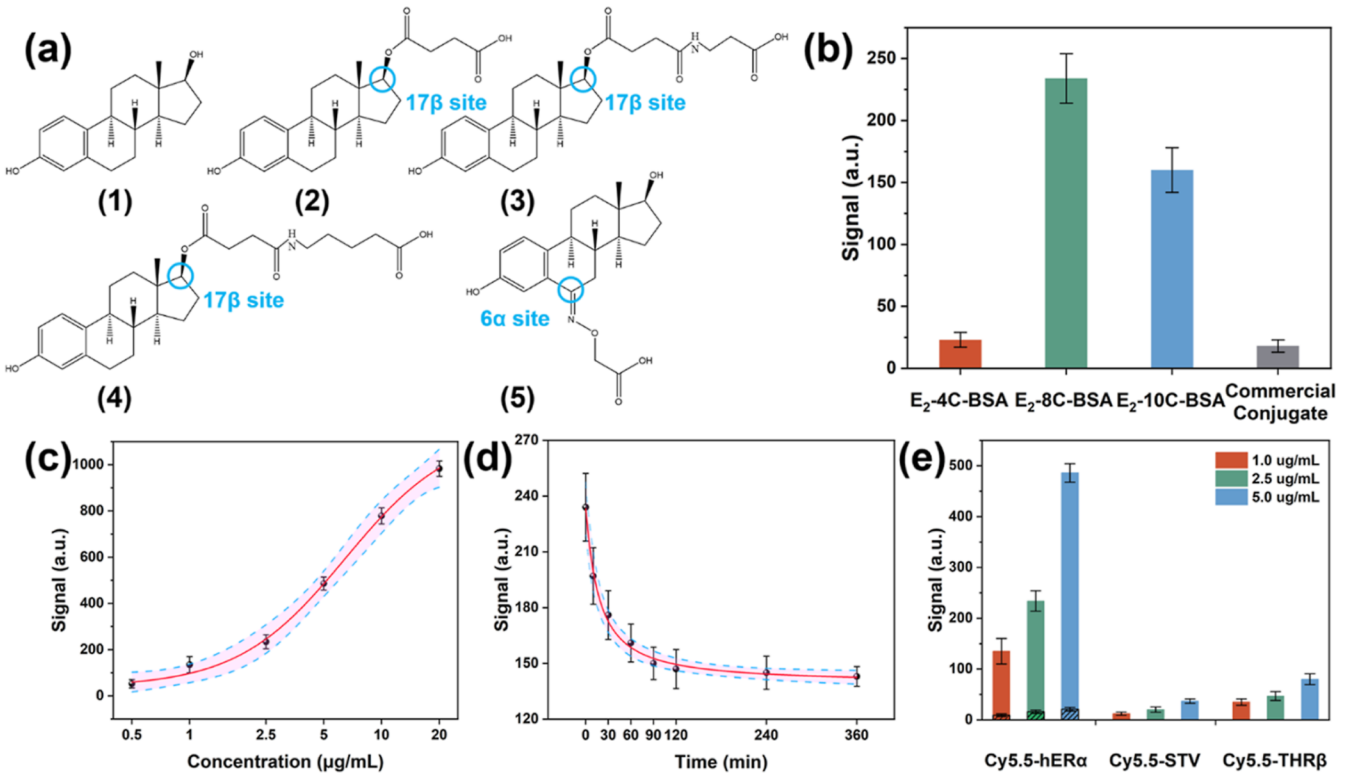

Figure 2. Optimization of experimental conditions. (a) Structures of (1) E_2, (2) E_2−4C, (3) E_2−8C, (4) E_2−10C, and (5) commercial conjugate β-estradiol 6-(*o*-carboxymethyl)oxime. The blue circles represent the derivative sites; (b) signal responses of E_2−4C−BSA, E_2−8C−BSA, E_2−10C−BSA, and commercial conjugate-functionalized optical fibers toward 2.5 μg/mL Cy5.5−hERα−LBD; signal responses at different (c) concentrations of Cy5.5−hERα−LBD; (d) binding time with 1000 ng/L E_2. Blue dotted lines represent the 95% confidence band; and (e) signal responses of E_2−8C−BSA−functionalized optical fiber (the shaded region represents the signal on the BSA−functionalized optical fiber) to different concentrations of Cy5.5−hERα−LBD, Cy5.5−STV, and Cy5.5−THRβ. All error bars correspond to the standard deviation (n = 3).

binding buffer to 5× water samples, which contained 1% methanol, for biosensor detection.

Statistical Analysis. Statistical analysis was performed with Origin 2021 software. Each experiment was conducted three times in parallel. The four-parameter logistic model with unfixed lower and upper boundaries was adopted for curve fitting. The results are presented as the measured mean value ± standard deviation. The error bars in all figures represent the standard deviations from three individual experiments.

RESULTS AND DISCUSSION

Sensing Mechanism. Figure 1a depicts an instrument photograph and a structure diagram of the custom-designed NR-based biosensor based on the evanescent wave fluorescence and bioaffinity assay setup. More details about the evanescent wave fluorescent system can be found in the Supporting Information (section S8). By screening estrogenic disruptors as a typical representative of EDCs, we designed the sensing mechanism shown in Figure 1b. More specifically, a concise indirect competition bioassay was established by introducing fluorescence-labeled NR and EDC-functionalized optical fibers. The Cy5.5−hERα−LBD conjugate consisting of a fluorescent dye and the hERα−LBD was synthesized as the biological recognition element to achieve receptor−ligand affinity interactions and signal excitation. The E_2−8C−BSA conjugate was designed and then functionalized on the optical fiber surface to ensure that the E_2 moiety on the surface exhibited recognition capability by optimizing the flexible linking arm that connected E_2 and BSA. When ligands from the estrogen receptor were present in the environmental waters (concentrated if necessary), the bioaffinity reaction of the Cy5.5−hERα−LBD conjugate and target compounds in the sample occurred within 90 min. Subsequently, the mix was fed into the flow cell of the optical fiber, resulting in competition between the E_2 moiety on the surface and the estrogenic disruptors to bind with the Cy5.5−hERα−LBD, which was accompanied by an increased fluorescent intensity under evanescent wave field excitation. The stronger the endocrine disrupting potential of environmental waters was, the weaker the fluorescent signal, resulting in a typical "turn-off" sensing mode. At the end of each measurement, washing buffer was applied to elute and regenerate the optical fiber for the next test cycle.

It is essential for the biosensor that hERa−LBD functions after the fluorescence labeling is performed. To confirm the binding activity, 20 μM hERα−LBD and Cy5.5−hERα−LBD were incubated with the E_2 standard solution for 90 min at room temperature at different molar ratios. The bound E_2 on hERα−LBD and Cy5.5−hERα−LBD was then eluted by using a methanol solution. The eluted E_2 was measured by HPLC and calculated by using the fitting curve of the peak area. As shown in Table S1, the retained ratios of E_2 under different conditions were calculated and found to decrease with an increasing molar ratio. We attributed the phenomenon to be the saturation of the estrogen receptor binding sites when E_2 was used in excess. When the reaction molar ratio of the estrogen receptor to E_2 was greater or equal to 1:1, the retained ratios of E_2 varied in the range from 80.64 to 91.27%

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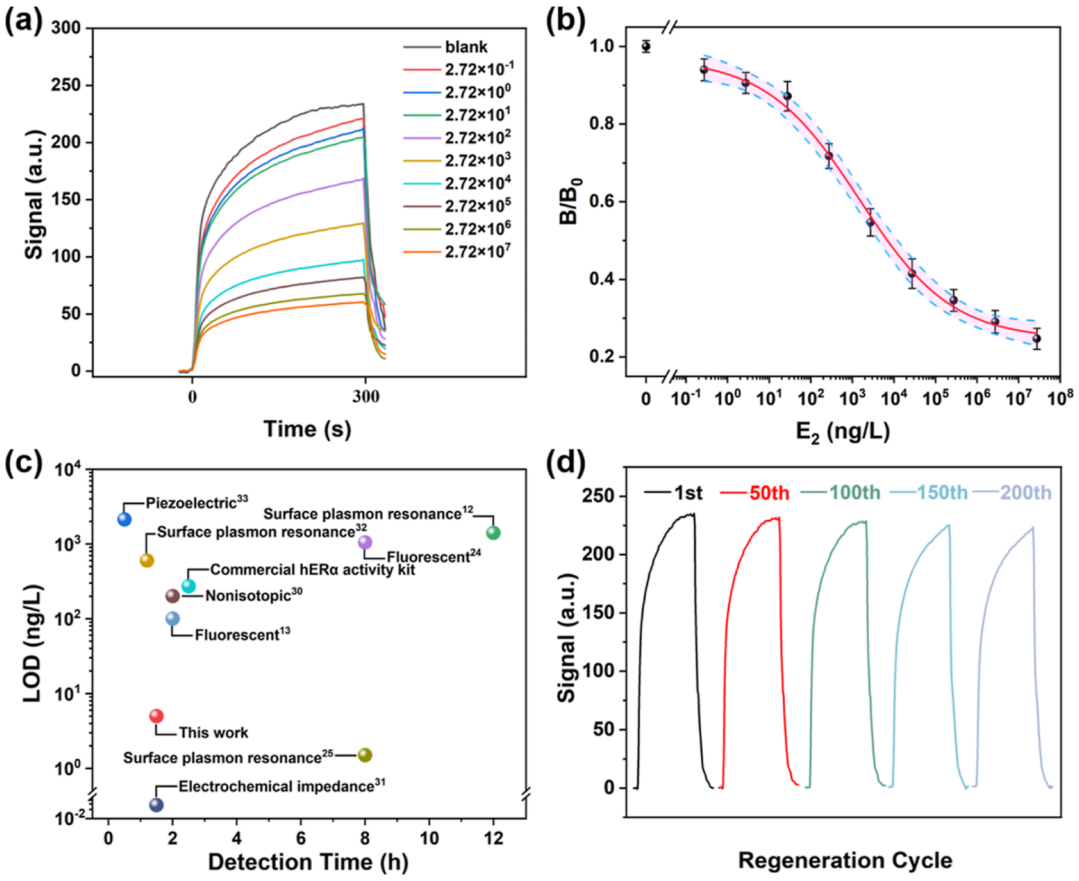

Figure 3. Performance evaluation of the developed biosensor. (a) Real-time signal responses without and with different E_2 concentrations ranging from 2.72×10^{-1} to 2.72×10^{7} ng/L, that is, 10^{-3} to 10^{5} nM, and (b) standard curve of the biosensor for E_2 detection. The blue dotted lines represent the 95% confidence band, and error bars correspond to standard deviation ($n = 3$), (c) comparison of the LOD and detection time of the biosensor with that of state-of-the-art estrogen receptor-based biosensors, and (d) signal regeneration of E_2−8C−BSA−functionalized fiber toward 2.5 μg/mL Cy5.5−hERα−LBD.

when both hERα−LBD and Cy5.5−hERα−LBD were used. These results indicated that the high binding activity and stable affinity capability of Cy5.5−hERα−LBD remained unchanged by labeling, providing the basis for the sensing strategy design.

The length and structure of the linking arms between E_2 and BSA are primary parameters that influence the capability of Cy5.5−hERα−LBD to recognize E_2 of the E_2−BSA conjugate that is immobilized on the solid surface. The rigid linking arms were excluded from the receptor−ligand affinity interaction due to the collision between the stiff linker structure and the ligand binding pocket.[28] Instead, estradiol derivatives with flexible carbon-framework linking arms, for example, E_2−*X*C (X = 4, 8, and 10) with the −COOH terminal [Figure 2a(2−4)] were designed to be coupled with BSA. When compared to the commercially available conjugate of β-estradiol 6-(*o*-carboxymethyl) oxime/BSA [Figure 2a(5)], the designed estradiol derivatives have the same 17β derivative site; thus, no impact on the receptor−ligand binding interaction was observed as reported previously.[29] As revealed by MALDI-TOF MS, the successful coupling of E_2−*X*C−BSA conjugates was achieved (Figure S5).

The obtained average coupling ratios of E_2−*X*C−BSA conjugates were calculated from the difference in molecular weight between BSA and E_2−*X*C−BSA conjugates. In our case, relatively high coupling ratios ranging from 11.9 to 13.5 for three designed estradiol derivatives were achieved (Table S2), and these derivatives were deemed effective for indirect competitive bioassay in the subsequent detection.[24] To select the optimal linking arm, the optical fiber functionalized with E_2−4C−BSA, E_2−8C−BSA, E_2−10C−BSA and the commercial conjugate β-estradiol 6-(*o*-carboxymethyl)oxime/BSA were incubated with 2.5 μg/mL Cy5.5−hERα−LBD, and the signals were recorded as shown in Figure 2b. The E_2−4C−BSA−functionalized optical fiber showed a negligible signal, probably because the linking arm was too short for the immobilized E_2 on the solid surface to enter the binding pocket of Cy5.5−hERα−LBD. E_2−8C−BSA exhibited the highest signal response. We attributed this result to the suitable active space provided by the eight-carbon linking arm, which facilitated the receptor−ligand binding interaction. However, the signal unexpectedly decreased when the linking arm was prolonged to 10 carbons, which may be associated with the weakened binding affinity between E_2−10C−BSA and Cy5.5−hERα−LBD due to the long linking arm. Notably, the commercial conjugate showed a weak signal response. The reported three-dimensional conformation of the hERα−LBD and E_2 complex showed that its E_2 aromatic ring (including the 6α derivative site) was completely encased within the predominantly hydrophobic cavity of hERα−LBD. A high number of hydrogen bonds was present, leaving the 17β phenolic hydroxyl of E_2 being exposed to the outside of the binding pocket.[8,9,28] Therefore, it could be inferred that the derivatization at the 6α site may damage the receptor−ligand

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Table 1. Performance Comparison of the Estrogen Receptor-Based Biosensors[a]

methods	linear region (ng/L)	LOD (ng/L)	detection time (h)	reusability (times)	refs
SPR	1400−14,000	1400	12	/	12
SPR	10−1000	1.5	8	/	25
SPR	500−10,000	600	1.2	/	32
fluorescent	100−20,000	100	2	/	13
fluorescent	/	/	12	300	23
fluorescent	20,800−476,700	1050	8	200	24
nonisotopic	/	200	2	/	30
electrochemical impedance	0.0272−272	0.0272	1.5	/	31
piezoelectric	2720−27,200	2120	0.5	30	33
fluorescent	40−51,325	5	1.5	200	this work

[a]"/" means undetectable or not available. "Ref." means the corresponding reference in the article.

specific interaction. As a result, E_2−8C−BSA was selected to modify the optical fiber and further optimize its performance.

Optimization of Experimental Conditions. For estrogen receptor-based biosensor that are based on the receptor−ligand affinity interaction, experimental conditions, such as the concentration of NR and binding time are critical parameters that affect the detection sensitivity. We tried to find the most sensitive range of this biosensor toward Cy5.5−hERα−LBD, and a higher sensitivity toward the targets was achieved. As depicted in Figure 2c, the signal response gradually increased with increasing concentrations of Cy5.5−hERα−LBD from 0.5 to 20 μg/mL. The linear region was fitted using the four-parameter logistic model from 2.5 to 10 μg/mL, that is, a total of 20−80% of the difference between the maximum and minimum signal response, which was the most sensitive range. To conserve reagent and hence reduce the test cost, 2.5 μg/mL was selected as the optimal concentration of Cy5.5−hERα−LBD to achieve high sensitivity. Moreover, the binding time between Cy5.5−hERα−LBD and E_2 was also an important factor. Figure 2d shows the influence of binding time on the signal responses of the E_2 assay. With the increase in binding time, additional external E_2 could bind to Cy5.5−hERα−LBD in the buffer solution; thus, the effect of indirect competition was stronger, causing the signal to decrease and basically reach saturation at 90 min. Hence, 90 min was identified as the optimum binding time to obtain high sensitivity. As described above, 2.5 μg/mL Cy5.5−hERα−LBD and a 90 min binding time were chosen and employed in subsequent experiments to detect the estrogenic binding activity.

It is widely acknowledged that non-specific adsorption is the main cause of false positive test results, particularly in complex environmental samples, decreasing the accuracy and sensitivity of the biosensor. The flexible conformational structure is prone to generate non-specific signals, especially for the NR-based biosensor. BSA is widely used as an inert protein to block surface non-specific binding sites in various biosensors. Generally, BSA is directly added either to ultrapure water or neutral buffer as a blocking solution to decrease the non-specific binding of the sensing surface. However, the poor blocking effect is considered to be insufficient.[27] In our case, a blocking buffer with a pH = 4.6 at the isoelectric point of BSA was used to block the fiber surface at 4 °C overnight so that the developed NR-based biosensor exhibited sufficient performances. In addition, binding buffer containing 5 mg/mL BSA was used to further eliminate the non-specific signal of receptors throughout the assay process. Consequently, the non-specific signals of the E_2−8C−BSA−functionalized optical fiber, which responded to both Cy5.5−labeled streptavidin (STV) and Cy5.5−labeled thyroid hormone receptor β (THRβ), and the BSA−functionalized optical fiber, which responded to Cy5.5−hERα−LBD, were negligible compared with the specific signals of Cy5.5−hERα−LBD at three concentration levels (Figure 2e), in which high signal−noise ratios greater than 10 were observed.

Evaluation of the Biosensor Performance. It is noteworthy that the estrogenic binding activity could be characterized using E_2-BAE because E_2 is an endogenous estrogen, there is a high content of E_2 in the body, and E_2 exhibits high binding affinity to the estrogen receptor. Under the optimal conditions described above, the real-time signal response of the biosensor was observed without and with various E_2 concentrations ranging from 2.72×10^{-1} to 2.72×10^{7} ng/L (Figure 3a). The signal of the blank sample was 235 with a relative standard deviation (RSD) of 8.6%, which corresponded well to the signal of 2.5 μg/mL Cy5.5−hERα−LBD as shown in Figure 2c. The dynamic signal changes clearly reflected the process of Cy5.5−hERα−LBD binding and dissociation on the fiber surface, and the fluorescence intensity gradually decreased with increasing E_2 concentration; this was due to the enhanced competitive binding of Cy5.5−hERα−LBD between the added external E_2 and the E_2−8C−BSA−functionalized optical fiber. To explore the quantitative relationship of the target concentrations and signal responses, the calibration curve was fitted with a four-parameter logistic model (Figure 3b). All data were the average of values measured three times in parallel with RSDs ranging from 7.2 to 9.3%, and satisfactory reproducibility was observed. The linear region was observed in the range of 40−51,324 ng/L, which was defined as 20−80% of the difference between the maximum and minimum signal response. In particular, according to the calculated rule of 90% of the signal difference, the limit of detection (LOD) could approach 5 ng/L with an RSD of 8.9% (n = 3), indicating that the biosensor exhibits a promising potential application in environmental monitoring and risk assessment.

To further demonstrate the analytical performance of the biosensor, the LOD and detection time for screening estrogenic binding activity were compared with those of previously reported estrogen receptor-based techniques, as shown in Figure 3c.[12,13,24,25,30−33] In addition, a more comprehensive comparison, including linear region and reusability, was obtained for the performance of estrogen receptor-based biosensors, is exhibited in Table 1. The preconcentration time was not included for the comparison because the process was not mentioned, except for refs 24 and 32. Clearly, the biosensor outperforms the majority of other

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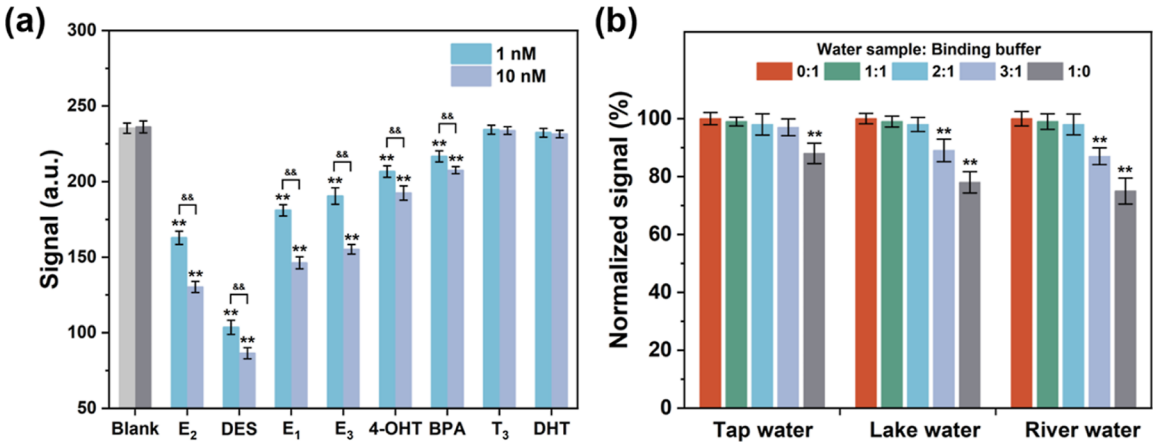

Figure 4. Reliability of the developed biosensor. (a) Signal responses of various compounds at two different concentrations (1 and 10 nM). The blank group was the signal of 2.5 μg/mL Cy5.5−hERα−LBD. The other groups were the signals of the corresponding compound that were incubated with the same concentration of Cy5.5−hERα−LBD. $^{**}p < 0.01$, compared with the blank group. $^{\&\&}p < 0.01$, compared with the signal of different concentrations of 1 and 10 nM and (b) matrix effects of different diluted ratios of water samples containing the final E_2 concentration of 1000 ng/L. $^{**}p < 0.01$, compared with the group of diluted ratios of 0:1.

biosensors (in both detection sensitivity and time) and commercial estrogenic binding activity kit that are based on estrogen receptor. It is worth mentioning that surface plasmon resonance (SPR) technology involves a rapid response to target binding when determining the binding affinity. However, to achieve the lower detection limit in analysis, the incubation time of E_2 and the estrogen receptor was usually overnight at 4 °C before SPR analysis,[12,25] which accounted for the large portion of the analysis time. In our system, we found that an incubation time of 90 min was ideal to achieve the signal plateau (Figure 2d), which explained the necessity of optimizing the incubation time. In particular, compared with our previous design,[23] this developed biosensor can overcome the time-consuming two-step bioaffinity conversion processes and cannot identify endogenous E_2. As expected, the estrogen receptor-based biosensor showed a significant improvement in that the detection time was shortened compared with that of the cell-dependent bioassays.[8−10] Considering that environmental endocrine disruptors are usually present at the ng/L level in natural environmental water samples, the developed biosensor can meet the demands of environmental monitoring.

The reusability of the sensing element is essential for the application of biosensors in environmental monitoring. A biosensor with good reusability means many advantages, such as low costs, fast detection times, and simple operation procedures. In this case, the E_2−8C−BSA−functionalized fiber sensing element can be regenerated by utilizing 0.5% SDS (pH 1.9) solution to break the reversible binding (noncovalent binding) between the receptor and its ligand and refresh the recognition capability of the E_2 moiety on the fiber surface. As depicted in Figure 3d, five representative signal traces were tested approximately every 50 times to evaluate the regeneration capability of this fiber sensing element. The fluorescence signals of the 50th, 100th, 150th, and 200th usages were 98, 97, 94, and 93% of the initial signal (the first), respectively, which indicated that the fiber sensing element exhibited excellent sensing performance even after at least 200 times uses. These results demonstrated that the developed biosensor exhibits satisfactory reusability and reliability for detecting estrogenic binding activity; thus, the biosensor exhibits considerable application potential in facile, automatic, and online environmental monitoring.

To evaluate the reliability of the developed biosensor, seven chemical compounds, including endogenous estrogens (E_1 and E_3), artificial estrogen agonist and antagonist drugs (DES and 4-OHT), typical phenolic compounds (BPA), and ligands of other NRs (T_3 and DHT) were incubated with Cy5.5−hERα−LBD. Then, the obtained solution was used for estrogenic binding activities assay. The E_2 group and the blank group (only the binding buffer did not contain other chemical compounds) were chosen as the control. Owing to the indirect competitive strategy, the stronger estrogenic-disrupting effect could lead to a weaker signal at the same concentrations. As shown in Figure 4a, compared with the three kinds of endogenous estrogens, DES exhibited the lowest fluorescence signal, which was 103 (taking the concentration of 1 nM as an example). E_2 is the most active endogenous estrogen, which expectedly yielded a lower fluorescence signal (162) than that of E_1 (180) and E_3 (190), which is consistent with previous results.[34−36] From observing the characteristics of the signal, it can be inferred that the estrogenic binding activities of these chemical compounds can be ordered as DES > E_2 > E_1 > E_3 > 4-OHT > BPA. In addition, T_3 and DHT exhibited comparable signals with those of the blank sample (235), indicating that the biosensor exhibited good recognition capability for compounds without estrogenic binding activity. Therefore, the developed biosensor not only possesses good selectivity but can also identify the strength of the disrupting effect. Thus, this biosensor exhibits potential in high-impact applications to assess the effects of environmental EDCs.

To prevent the influence of matrix effects, three environmental samples (tap water, lake water, and river water) were diluted with binding buffer before being measured. The results are shown in Figure 4b. In these experiments, E_2 was spiked with a final concentration of 1000 ng/L, which is close to the point of inflection (Figure 3b) and thus approximately reflects the EC_{50} of the assay. The measured signals in tap water, lake water, and river water were normalized to the signal measured in the spiked binding buffer (equal to 100%). A drop in the normalized signal indicates that the sample matrix interfered with the detection mechanism of the proposed biosensor.

https://doi.org/10.1021/acs.est.2c01323
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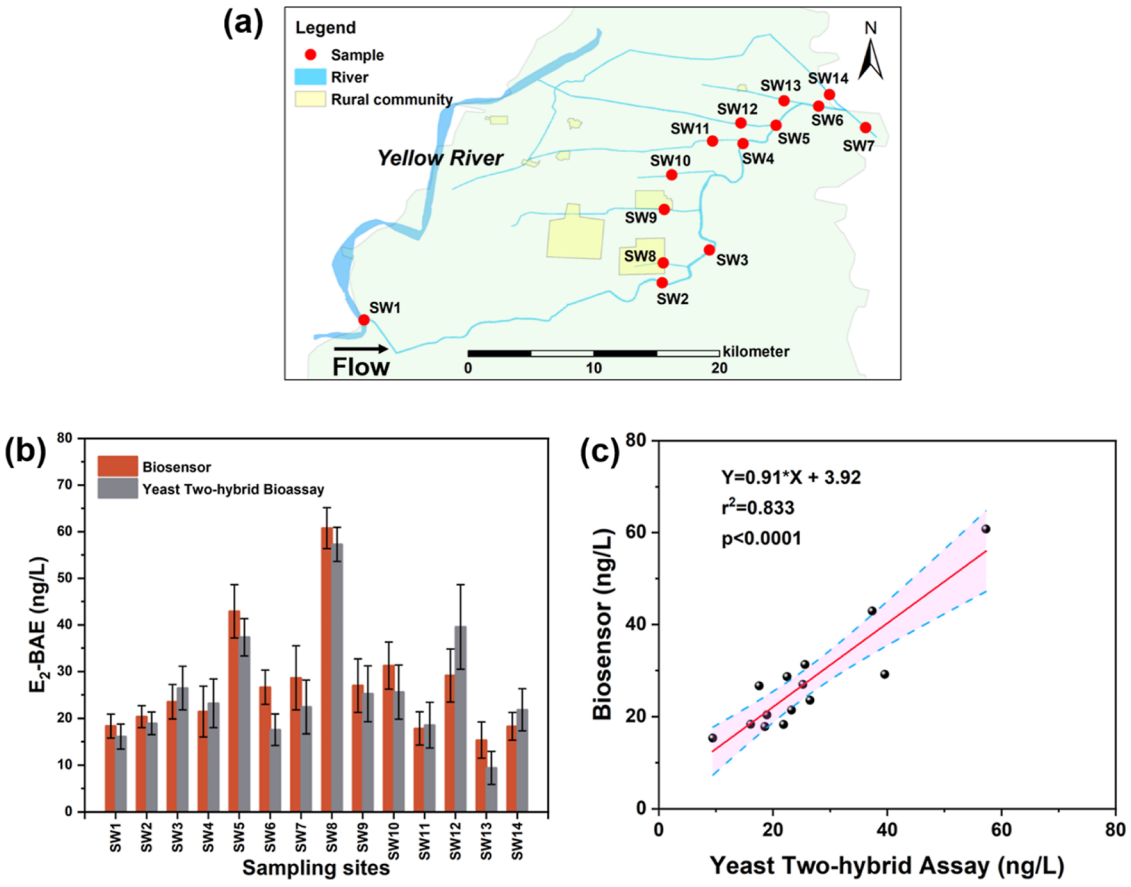

Figure 5. Applicability of the developed biosensor for detecting surface waters. (a) Surface waters in a typical rural community in the Yellow River basin in China and the distribution of sampling sites (SW1−14 represent surface water at different river channels). (b) E_2-BAE of 14 sampling sites detected using the biosensor and yeast two-hybrid bioassay. Error bars correspond to the standard deviation (n = 3). (c) Correlation analysis between the E_2-BAE of the yeast two-hybrid bioassay and the developed biosensor for 14 sampling sites in the rural community.

Notably, the environmental samples were tested using the biosensor, and the ligands with estrogenic binding activity were not detected before spiking with E_2. The interferences of matrix effects were negligible with different dilution ratios in tap water (water sample/buffer = 3:1), lake water, and river water (water sample/buffer = 2:1). To further confirm the applicability of the developed biosensor in environmental water samples, three real water samples (tap water, lake water, and river water) with five spiked concentrations of E_2 (from 1 × 10^1 to 1 × 10^4 ng/L) were assayed at the aboveoptimized dilution ratio. As shown in Table S3, the average recoveries ranged from 89 to 112% with an allowable RSD of less than 11.2% (n = 3). These results indicated that this proposed biosensor exhibits for detecting potential environmental estrogenic binding activities in real environmental water samples.

Generally, one mode of action of endocrine disruptors is the specific binding of a NR, which might result in interference with the normal synthesis and degradation of natural hormones. In theory, the developed biosensor can also be expected to exhibit excellent analytical performance in detecting a variety of environmental endocrine disruptors by replacing the corresponding NR. As described in the introduction, ONRs are a type of NR without ligands, or the ligands have not been found; therefore, ligands are not within the scope of biological recognition elements for the proposed biosensor. The results above indicated that hERα−LBD, as a typical SNR, is extremely applicable to this biosensor. Hence, THRβ, a classic non-SNR, was considered to demonstrate the universality of the biosensor. The detailed experimental procedure and sensing mechanism are similar to the above endocrine-disrupting chemicals assay. Similarly, the thyroid hormone binding activity could be characterized by T_3-BAE. As shown in Figure S6a, 2.5 μg/mL Cy5.5-THRβ, as an optimized concentration, can meet the requirement of detecting thyroid hormone binding activity. A linear region was observed in the range of 417−134,197 ng/L (Figure S6b), and the LOD was calculated to be 93 ng/L with an RSD of 9.7% (n = 3). Therefore, it can be considered that the developed biosensor exhibits good universality for other environmental EDCs.

Mapping Estrogenic Binding Activity of Surface Waters in a Rural Community. To validate the practicability of the developed biosensor in environmental water samples, the risk of the unknown environmental estrogenic binding activities of surface waters was assessed in the sample area from a typical rural community in the Yellow River basin in China. The details of the sampling sites are described below the map (Figure 5a), and the various surface waters (SW1−14) were expected to be representative of the range of conditions in the rural community environment. The total concentration of estrogenic-disrupting chemicals in the surface waters could be calculated according to the standard qualification curve of E_2. As shown in Figure 5b, the E_2-BAEs of various sampling sites

https://doi.org/10.1021/acs.est.2c01323
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were measured using both the developed biosensor and yeast two-hybrid bioassay, and all results were in the range of 10−70 ng/L. Among these sampling sites, the E_2-BAEs of SW5, SW8, and SW12 were slightly higher than those of the other sampling points. The sampling site SW8 was located inside a rural community, and the high concentration might be caused by the uncontrolled discharge of domestic sewage considering that the average collection and treatment ratio in Chinese rural communities is below 25%. Moreover, several family farms such as duck and hog farms were located in the upstream of sampling sites SW5 and SW12. The urine and feces excreted by the ducks and hogs might contribute to the high E_2-BAE. Compared with the worldwide reported E_2-BAE of surface waters in river basins, which ranged from 0 to 120 ng/L,[37,38] these sampling sites exhibited the intermediate level. In addition, a strong linear relationship (r^2 = 0.833 and p < 0.0001) of the results measured using the developed biosensor and the yeast two-hybrid bioassay was observed for all 14 sampling sites (Figure 5c), indicating the excellent accuracy and reliability of this technology in real applications.

In summary, we developed a NR-based biosensor for the sensitive quantification of broad-ranging EDCs in environmental water samples. Fluorescence−labeled NRs acted simultaneously as signal recognition and signal report. Based on a competitive bioassay strategy that is concise and indirect, the proposed method for screening estrogenic and thyroid hormone-disrupting potential showed satisfactory performance within a wide linear range from 40 to 51,325 ng/L in E_2-BAE and 417 to 134,197 ng/L in T_3-BAE. A satisfactory LOD of 5 ng/L for estrogens and 93 ng/L for thyroid hormone disruptors was achieved within 90 min. By mapping the estrogenic binding activity of surface waters from a rural community in the Yellow River basin, China, a high correlation was observed between the obtained results and those of the traditional yeast two-hybrid bioassay. In addition, the affinity-based biosensor exhibited good universality and versatility for various EDCs by changing diverse NRs as biological recognition elements. The biosensor can potentially transform the landscape of environmental monitoring technologies and provide a foundation for expansive development centered around this technology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c01323.

Several related materials and reagents; structure and classification of endocrine-related NRs and ligands; construction and characterization of recombinant hERα-LBD; synthesis procedure of fluorescent labeling hERα−LBD; synthesis procedure of estradiol derivative−BSA conjugates; preparation process of optical fiber sensing elements; yeast two-hybrid bioassay of EDCs; evanescent wave fluorescent biosensors; Cy5.5−hERα−LBD activity verification; estradiol derivative−BSA conjugates identification; coupling ratios of estradiol derivative−BSA conjugates; and matrix effects in three water samples and universality of the developed biosensor (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This project was supported by the National Key Research and Development Project (2019YFD1100505), the Tsinghua University Initiative Scientific Research Program (2019Z02CAU), the Special fund of State Key Joint Laboratory of Environment Simulation and Pollution Control (21K06ESPCT), and the National Nature Science Foundation (21976100).

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