Cyanine-based colorimetric and fluorescent probe for the selective detection of diethylstilbestrol in seawater, shrimp and fish samples

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ABSTRACT

The synthetic estrogen drug diethylstilbestrol (DES) plays important roles in the treatment of estrogen deficiency disorders for human beings. The excessive intakes of DES can lead to physiological dysfunction and raise the risk of ovarian cancer, breast cancer and other diseases. However, it is still abused for improving the fat deposition or sex reversal procedure in aquatic economic creatures for pursuit of huge interests. DES can exist in aquatic products or polluted aquacultural seawater, which seriously threatens human health. Therefore, it is desirable to establish simple and sensitive methods for the detection of DES. In this work, we have developed a new colorimetric and fluorescent probe Cy-DES for the detection of DES with high sensitivity and selectivity. As a near-infrared probe, Cy-DES is able to avoid autofluorescence of dissolved organic compounds and maximize signal-to-background contrast. Taking advantage of the strong electrostatic interaction between the probe Cy-DES and DES, the spectroscopic properties of probe Cy-DES can be obviously changed in presence of DES. Under testing conditions, there is an excellent linearity within the range of 1–8 μM ($r = 0.9997$) and the detection limit is 0.2 μM. The probe Cy-DES is successfully applied for the detection of DES in spiked seawater, shrimp and fish samples. Additionally, the detection of DES can be directly achieved by naked eyes with the utilizing of probe Cy-DES. The developed method is of great potential for application in the on-site detection of DES.

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1. Introduction

As one of the synthetic estrogen drugs, diethylstilbestrol (DES) is usually employed to the prevention and treatment of pregnancy complications [1]. The physiological role of DES is similar with the natural estrogen, but DES is more stable and can remain in human body for a much longer time. Recently, the toxicological and carcinogenic properties of DES to animals and human beings have caused widespread concern. After being excessively exposed to DES, the male mice increase the risk of reproductive tract lesions and immunological dysfunctions [2–4]. Long-term intake of DES also raises the possibility of ovarian and breast cancers due to its genotoxicity [5–7]. However, DES can improve the fat deposition and quality of meat as a growth promotant [8,9]. And it is still commonly abused on growth and survival parameters or sex reversal procedure in mariculture engineering for pursuit of huge economic interests [10]. DES may exist in aquatic products or run into the

water by urine and feces, which has seriously threatened human health. Therefore, it is desirable to develop simple and direct methods for the detection of DES.

To date, several methods have been established to detect DES, such as high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), electrochemical techniques, and chemiluminescence [11–18]. HPLC can detect DES with good sensitivity and selectivity, but it usually needs sophisticated sample pretreatment [11,12]. ELISA is a quite sensitive approach, but preparation of antibody is time-consuming and troublesome [13–15]. The electrochemical techniques and chemiluminescence show low detection limit with good selectivity, but usually interfered by complex matrices [16–18]. Compared with these methods, fluorescence methods are outstanding due to its excellent sensitivity and selectivity. And colorimetric methods are much simpler and are able to be recognized by naked eyes. The two mentioned methods can be easily utilized in the on-site test [19,20]. However, to date, few colorimetric and fluorescence methods have been developed for detecting DES, especially the near-infrared (NIR) probes, which can avoid background fluorescence of dissolved organic compounds in natural seawater and bio-samples

[21]. In this work, we developed a NIR probe Cy-DES to sensitively and selectively detect DES in both colorimetry and fluorescence method. The typical NIR dye heptamethine cyanine is chosen as the fluorophore, which has drawn great interest because of its high molar extinction coefficient and low cytotoxicity. In presence of DES, the self-aggregation of probe Cy-DES occurs, which leads to obvious changes in its spectroscopic properties including UV–Vis absorption spectra and fluorescence spectra. Take this advantage, the probe Cy-DES can successfully detect DES in complex matrices such as seawater, shrimp and fish samples. As a simple, sensitive, and nake-eye recognizable probe, Cy-DES may be conveniently utilized to detect DES on-site.

2. Experimental

2.1. Chemicals and instruments

All the chemicals used in the experiments were analytical reagent grade. UV–Vis spectra were measured on a μ-Quant microplate reader Nanodrop 2000C (Thermo Scientific, USA) with a 1 cm quartz cell. Fluorescence spectra were quantitatively measured by FluoroMax-4 spectrofluorometer with a xenon lamp and 1 cm quartz cells. Cytotoxicity assay was carried out with a microplate reader (TECAN Infinite 200). High-resolution mass spectra were carried on LCQ Fleet LC–MS System (Thermo Fisher Scientific). ^{1}H NMR and ^{13}C NMR spectra were carried on a Bruker spectrometer.

2.2. Preparation of the probe Cy-DES

Ketone-Cy (100 mg, 0.19 mmol) was synthesized in our laboratory [22] and triethylamine (278 μL, 0.6 mmol) was dissolved in 15 mL anhydrous CH_2Cl_2 at 0 °C. Then propionyl chloride (16.5 mL, 0.19 mmol) was added dropwise during 30 min (Scheme S1). The mixture was stirred 24 h at 25 °C. After evaporated in vacuo, the crude product was obtained as a deep green solid. Finally, the probe Cy-DES was isolated by silica chromatography eluted with CH_2Cl_2/CH_3OH (4:1, v/v) as a green solid (35 mg, yield 31%). ^{1}H NMR (500 MHz, DMSO) δ 8.28–8.25 (d, 1H), 7.61–7.59 (m, 2H), 7.44 (m, 1H), 7.27 (m, 2H), 6.35–6.32 (d, 1H), 6.25–6.22 (d, 1H), 4.24–4.22(s, 4H), 4.05–4.00 (m, 2H), 2.91–2.89 (m, 4H), 2.73–2.67 (d, 2H), 1.99 (s, 6H), 1.31–1.16 (m, 17H). ^{13}C NMR (126 MHz, DMSO) δ 203.18, 195.65, 179.12, 174.43, 172.06, 171.51, 170.78, 158.94, 153.23, 142.13, 141.54, 139.63, 129.11, 125.47, 122.97, 121.62, 111.67, 100.91, 60.07, 49.04, 48.86, 27.71, 24.28, 21.19, 20.95, 14.60, 12.66, 9.64. LC–MS (ESI+): m/z $C_{37}H_{45}N_2O_2^+$ calculated. 549.3476, found [M$^+$] 549.3473. Elemental analysis calculated (%) for $C_{37}H_{45}N_2O_2^+$: C, 80.9; H, 8.2; N, 5.1 O, 5.8; found: C, 80.9; H, 8.3; N, 5.1; O, 5.7.

2.3. Detection of DES by probe Cy-DES

All absorption and fluorescence spectra were detected in 1.0-cm cuvette cells. Before measurement, 1 μM probe Cy-DES was incubated with different concentrations of DES in 50 mM HEPES buffer at 37 °C for 15 min. The absorption spectra of Cy-DES were detected from 350 to 850 nm. The fluorescence spectra of Cy-DES were detected from 740 to 880 nm with excitation at 720 nm.

2.4. Detection of DES in real samples

Standard seawater samples were purchased from China Second Institute of Oceanography, State Oceanic Administration. Before UV–Vis detection, seawater samples were filtered through 0.22 μm membrane to remove particulate matters. Shrimp and fish samples were pretreated according to the literature [23]. 10 g shrimp and fish samples were added to 100 mL containers. And 50 mL

methanol was added to the containers. The samples were extracted by ultrasonic for 1 h after adequate shaking. And supernatants were collected by centrifuging at 10,000 rpm for 5 min. After evaporation, the residue was dissolved in 1 mL of methanol and the solution was diluted to 50 mL with ultrapure water. Then the pre-treated seawater, shrimp and fish samples were spiked with different concentrations of DES and detected by our probe and high performance liquid chromatography (HPLC) method. The HPLC method for detection of DES was carried out with the mobile phase consisting acetonitrile–water (70:30, v/v) at a flow rate 1.0 mL/min. The wavelength used to measure DES was set at 241 nm [12].
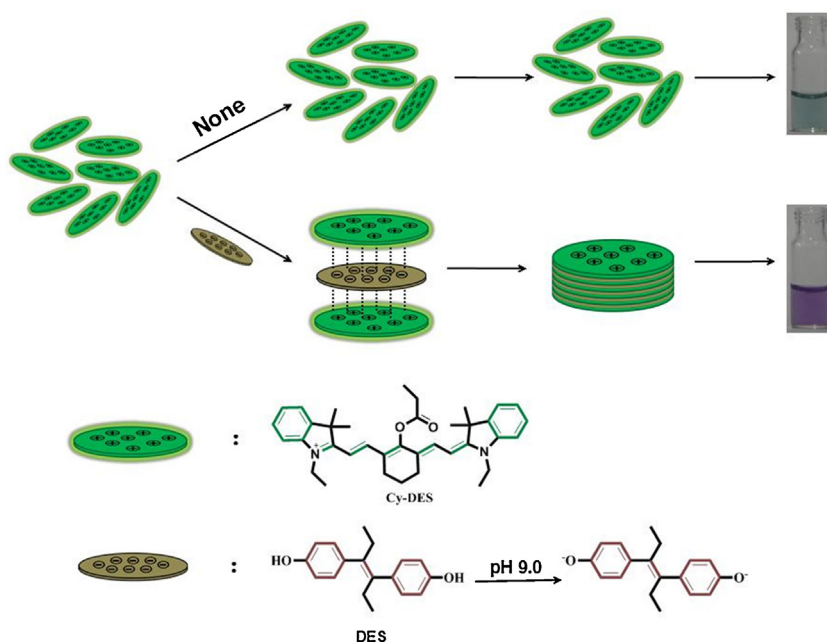
3. Results and discussion

3.1. Probe design and proposed mechanism

We designed and synthesized a colorimetric and near-infrared (NIR) fluorescent probe Cy-DES for the rapid and selective detection of DES (Scheme 1). The probe Cy-DES was developed by equipping heptamethine cyanine with propionyl chloride. After equipped with propionyl chloride, the probe's emission wavelength was centered at 790 nm (Fig. S1), because the heptamethine cyanine changed from ketone form to enol form and delocalized the positive charge by resonance over both nitrogen atoms (Schemes 1 and S1) [24,25]. This NIR probe Cy-DES was more optimal for detecting DES in seawater and bio-samples with its outstanding advantage of avoiding background fluorescence of other dissolved organic compounds. We anticipate that the conjugated system of DES delocalized the negative charge by acrossing both phenolic hydroxyl under alkaline environment [26]. The intermolecular aggregation occurs due to electrostatic interaction between cyanine dyes and DES, which leads to changes in the spectral properties of probe Cy-DES including the fluorescence quenching and the absorption hypsochromic shift [27,28].

However, the absorption hypsochromic shift of cyanine dye can also be induced by the modulation of intramolecular polymethine π-electron system with the cleavage of the ester bond [22,29] To verify the proposed mechanism of the probe Cy-DES for the detection of DES, more experiments were performed from different aspects. As shown in Fig. S2, the absorption spectrum of Cy-DES did not change in the presence of 1 mM phenol, which might cleave the ester bond of Cy-DES by its nucleophilic characteristic. The results indicated that the fluorescence/UV spectra changes of probe Cy-DES were not induced by phenolic hydroxyl. After the probe Cy-DES incubated with 10 μM DES at 30 °C for 20 min, the emission peak of ketone-Cy did not emerge, which further verified that the ester bond of probe Cy-DES could not be cleaved by phenolic hydroxyl. As shown in Fig. S3, the intermolecular aggregation of Cy-DES in absence/presence of DES was clearly observed from the images collected by the optical microscopy [30]. Therefore, the detection mechanism of DES by probe Cy-DES was not caused by the modulation of intramolecular polymethine π-electron system but by the intermolecular aggregation. Considering that the structure of Cy-DES and DES are both in-plane charge-separation, the aggregation of Cy-DES may be similar to the H-aggregates of cyanine dye [31–33], which forms as a plane-to-plane stacking and is characterized by an obvious hypsochromically shifted absorption band (Scheme 1). To further investigate the fluorescence quenching mechanism, the fluorescence intensity quenching data was handled referred to Stern–Volmer (SV) equation:

$$\frac{I_0}{I} = 1 + K_{sv}[Q]$$

where I/I_0 meant the fluorescence intensity of Cy-DES with/without DES, respectively. And K_{sv} meant the quenching constant. As shown in Fig. 1, the SV plots were nearly linear at low DES concentrations,

Scheme 1. Proposed mechanism of probe Cy-DES for the detection of DES.

which began to diverge from linearity and bended upwards with the concentration increasing. The results indicated a combination of dynamic and static quenching within the intermolecular aggregation of Cy-DES in presence of DES [34]. The quenching constant $K_{SV} = 1.3 \times 10^5$ by the fitting of the SV plot, which demonstrated a high-quenching ability of DES to probe Cy-DES.

3.2. The detection of DES by probe Cy-DES

To test the viability of probe Cy-DES, the optimal condition was firstly investigated. As shown in Fig. S4, the best performance of probe Cy-DES for the detection of DES was in 50 mM B-R solutions, pH 9.0 and incubated at 30 °C for 20 min. Nextly, probe Cy-DES

Fig. 1. The Stern–Volmer equation plot of Cy-DES in the presence of DES. Our probe Cy-DES (1 μM) with different concentrations of DES (0, 1.0, 2.0, 4.0, 6.0, 8.0 and 10 μM) in 50 mM B-R solutions, pH 9.0 and incubated at 30 °C for 20 min. Data were the means for three independent experiments.

with various concentrations of DES (0, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 15 and 20 μM) was performed under this test condition. As shown in Fig. 2a, the UV–Vis absorption of probe Cy-DES was centered at 785 nm, which displayed a green color. In the presence of different concentrations of DES, the absorption peak at 785 nm decreased gradually and a new absorption peak at 550 nm raised with a large hypsochromic-shift of 235 nm, which indicated the intermolecular aggregation of Cy-DES. And the color of Cy-DES changed from green to purple, which can be clearly recognized by naked eyes. The probe Cy-DES showed strong fluorescence intensity at 790 nm, a near infrared region. After DES added into the solution, a remarkable fluorescence intensity decrease was observed and the fluorescence quenching efficiency of probe Cy-DES increased gradually to 78%, which indicated the strong electrostatic interactions between probe Cy-DES and DES after intermolecular aggregation (Fig. 2c). At pH 9.0, the DES was mainly in ionic form because of the dissociation of phenolic hydroxyl groups and the negative charge was delocalized in the conjugated system [35]. Therefore, the probe Cy-DES with positive charge was easily attracted by DES and intermolecular aggregation occurred, which induced obvious changes of Cy-DES spectral properties. As shown in Fig. 2b and d, both the UV–Vis absorption and the fluorescence intensity of Cy-DES were linearly proportional ($r = 0.9997$) to the DES concentrations from 1 to 8 μM because the colorimetric signals accompanied with fluorescent signals were both generated by the intermolecular aggregation between Cy-DES and DES. And the detection limit (LOD) for DES was 0.2 μM (signal-to-noise (S/N) ratio = 3), which was similar with the HPLC method by solid-phase extraction for the analysis of diethylstilbestrol (LOD = 0.22 μM) [23] and the ELISA method by metal ions-based immunosensor for determination of diethylstilbestrol (LOD = 0.38 μM) [14]. We also investigated the reversibility of probe Cy-DES in the detection of DES. The reversibility of the intermolecular aggregation of heptamethine cyanine usually depends on environmental parameters especially pH [27]. However, the intermolecular aggregation of probe DES in the presence of DES was not reversible by regulating pH values, which probably due to the strong intermolecular forces between probe Cy-DES and DES. Additionally, we estimated the cytotoxicity of Cy-NB by MTT assays with the help of HeLa, HepG2, and MCF-7 cells. As shown in Fig. S6, the
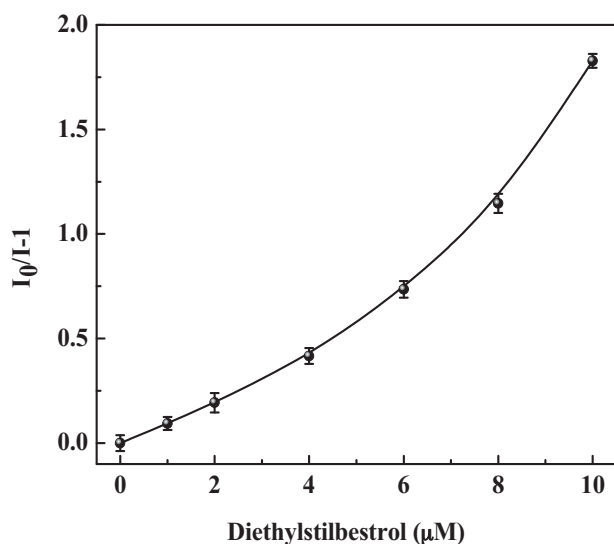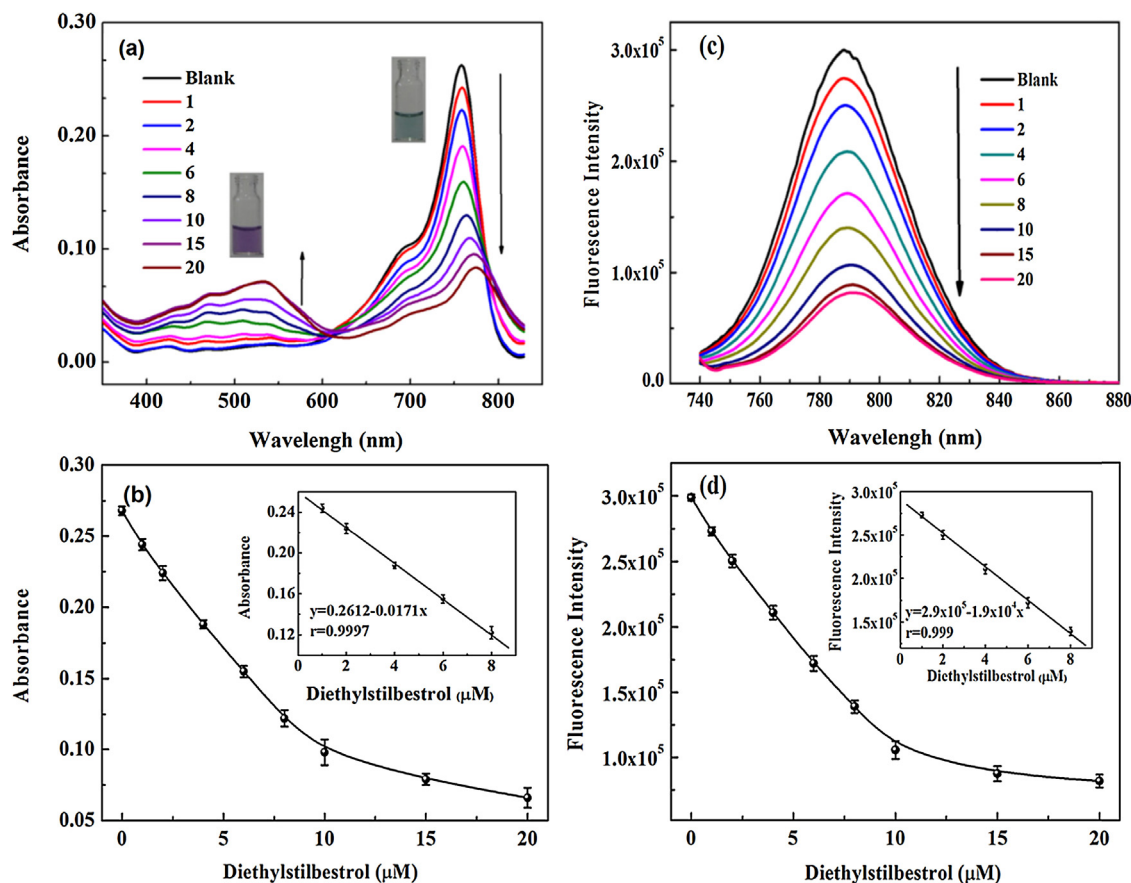
Fig. 2. Determination of DES by Cy-DES by absorption spectra and fluorescent emission spectra. (a) Absorption spectra of analyzing different concentrations of DES: 0, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 15 and 20 μM. (b) Determination of DES utilizing Cy-DES by colorimetric analysis (insert: liner regression equation, $y = 0.2612 - 0.0171x$, $r = 0.9997$ where "A" represents the absorbance of Cy-DES at 785 nm and "x" means the substrate concentration). (c) Fluorescent emission spectra of analyzing different concentrations of DES: 0, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 15 and 20 μM. (d) Determination of DES utilizing Cy-DES by fluorescent analysis (insert: linear regression equation, $y = 2.913 \times 10^5 - 1.927 \times 10^4$, $r = 0.9997$ where "y" represents the fluorescence of Cy-DES and "x" represents the substrate concentration). Data are the means for three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cellular viability was almost 99% in the presence of 1 μM Cy-DES, which indicated the low cytotoxicity of probe Cy-DES. All above results indicated that our colorimetric and fluorescent probe Cy-DES can be utilized to detect DES with high sensitivity, which are expected to achieve the simple and sensitive detection of DES in real water and bio-samples.

3.3. Selectivity of Cy-DES to DES

To evaluate the selectivity of the probe Cy-DES toward DES, the fluorescence and absorption spectra response of the DES as well as possible interferences including estrogens, metal ions and fish drugs were taken into consideration. To examine the interference of other estrogens whose structure were quite similar with DES, the probe Cy-DES was incubated with 10 μM DES, 10 μM bisphenol A, 100 μM estrone, 17-α-estradiol, β-estradiol and estriol, respectively. As shown in Fig. 3a, our probe Cy-DES showed a good selectivity toward DES over these estrogens. Fish drugs are important interferences in the detection of DES as they are commonly used for disease management in mariculture engineering. To investigate the selectivity of Cy-DES to DES over fish drugs, the probe Cy-DES was incubated with 10 μM DES, 100 μM furazolidone, sulfapyridine, sulfadimidine, sulfadoxine, trichlorphon, sulfathiazole, bromophos methyl, bromophos ethyl and diazinon, respectively. As shown in Fig. 3b, our probe Cy-DES showed an excellent selectivity toward DES over these fish drugs. The detection of DES

concentration in aquaculture seawater was an important mission in the design of probe Cy-DES. To achieve this aim, the detection of DES by probe Cy-DES should not be influenced by high salinity. Therefore, we examined the influence of metal ions on the detection of DES by probe Cy-DES. The probe Cy-DES was incubated with 10 μM DES in 50 mM B-R solutions and 10 μM DES with 0.5 M Na$^+$, 100 μM K$^+$, Li$^+$, Ca^{2+}, Cd^{2+}, Mg^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Mn^{2+}, respectively. As shown in Fig. 3c, the detection of DES by probe Cy-DES would not be influenced by these metal ions. Additionally, we also verified the selectivity of probe Cy-DES to DES on 96-well microtiter plates. As shown in Fig. 3d, DES could be easily picked out from other potential interferences in presence of probe Cy-DES. All above results indicated that our probe Cy-DES owned an excellent selectivity toward DES over other interferences.

3.4. Detection of DES in real samples

To study the matrix effect on the detection of DES by probe Cy-DES, seawater, shrimp and fish samples spiked with 2.0, 4.0, 8.0 μM DES were tested. The seawater samples were first filtered to remove insoluble substances. However, the dissolved organic compounds including amino acids, sugar acids, carboxylic acids and fatty acids were still existed in the natural seawater, whose fluorescence emission spectra were usually located from 300 to 400 nm [21]. Therefore, the fluorescence probes with a shorter emission wavelength are easy to be influenced by these dissolved organic
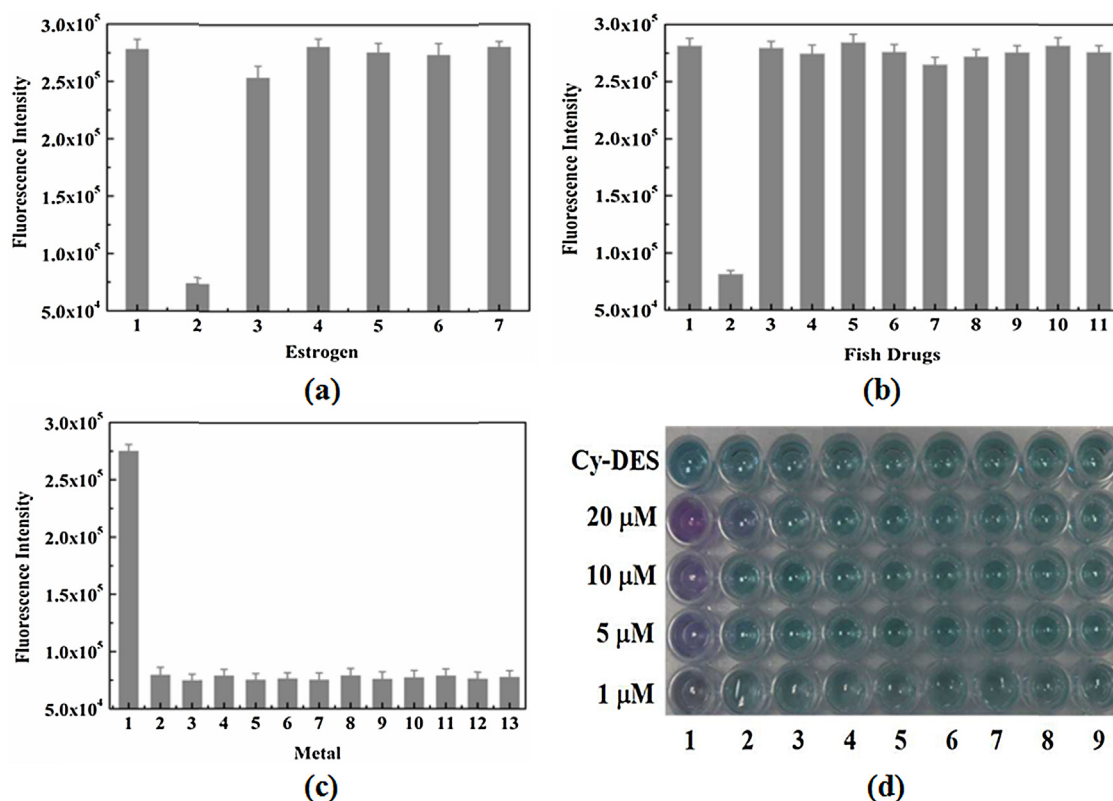
Fig. 3. The selectivity of Cy-DES to DES over estrogens, fish drugs and metal ions. (a) 1–7 means control (Cy-DES along), 10 μM DES, 10 μM bisphenol A, 100 μM estrone, 17-α-estradiol, β-estradiol and estriol, respectively; (b) 1–11 means control (Cy-DES along), 10 μM DES, 100 μM furazolidone, sulfapyridine, sulfadimidine, sulfadoxine, trichlorphon, sulfathiazole, bromophos methyl, bromophos ethyl and diazinon, respectively; (c) 1–13 means control (Cy-DES along), 10 μM DES, 10 μM DES with 0.5 mM Na⁺, 100 μM K⁺, Li⁺, Ca²⁺, Cd²⁺, Mg²⁺, Pb²⁺, Cu²⁺, Zn²⁺, Hg²⁺ and Mn²⁺, respectively; (d) 1–9 means DES, bisphenol A, estrone, 17-α-estradiol, β-estradiol, furazolidone, sulfapyridine, sulfathiazole, and diazinon. Data are the means for three independent experiments.

Table 1
The results obtained by Cy-DES and HPLC for the detection of DES.

Sample	Spiked (μM)	This method (μM)	Recovery (%)	HPLC (μM)	Recovery (%)
Seawater	0.0	ND	ND	ND	ND
	2.0	1.96 ± 0.14	98.0 ± 7.0	2.08 ± 0.09	104.0 ± 4.5
	4.0	3.68 ± 0.27	92.0 ± 6.8	3.87 ± 0.21	96.8 ± 5.2
	8.0	8.16 ± 0.51	102.0 ± 6.4	7.85 ± 0.37	98.1 ± 4.6
Shrimp	0.0	ND	ND	ND	ND
	2.0	1.86 ± 0.21	93.0 ± 10.5	1.97 ± 0.13	98.5 ± 6.5
	4.0	4.17 ± 0.28	104.2 ± 7.0	4.21 ± 0.19	105.2 ± 4.7
	8.0	7.69 ± 0.63	96.1 ± 7.9	8.19 ± 0.57	102.4 ± 7.1
Fish	0.0	ND	ND	ND	ND
	2.0	2.13 ± 0.09	106.5 ± 4.5	1.89 ± 0.07	94.5 ± 3.5
	4.0	4.19 ± 0.36	104.7 ± 9.0	3.89 ± 0.31	97.2 ± 7.7
	8.0	8.28 ± 0.46	103.4 ± 5.8	7.89 ± 0.57	98.6 ± 7.1

ND means not detectable.

compounds. As shown in Table 1, the detected concentrations of DES by probe Cy-DES were 1.96, 3.68 and 8.16 μM, respectively, which were approximate to the values of 2.08, 3.87 and 7.85 μM detected by HPLC, respectively. The results of the present colorimetric and fluorescent probe Cy-DES were in excellent agreement with those obtained by HPLC, which indicated that the probe Cy-DES was suitable for the direct detection of DES concentration in seawater samples. And the detection of DES by Cy-DES would not be influenced by dissolved organic compounds in seawater, which benefited from its NIR property. In the shrimp and fish samples, the DES in these samples could also be successfully detected by Cy-DES and the results agreed well with those obtained by the traditional method HPLC (Table 1), which indicated that the residual DES in aquatic products could be well detected by the developed

probe Cy-DES. All these results verified that our probe Cy-DES was credible and practically feasible for detecting DES in real samples.

4. Conclusions

In summary, we have developed a new and simple colorimetric and fluorescent probe Cy-DES for the selectively detection of DES. The probe is established based on the intermolecular aggregates between Cy-DES and DES by strong electrostatic interaction. In the presence of DES, an obvious absorption hypsochromic-shift of Cy-DES occurs and the color of the solution changes from blue to purple. As a result, the probe can conveniently detect DES by the naked eyes. The detection of DES by probe Cy-DES cannot be

influenced by high salinity. As a kind of NIR probe, the probe Cy-DES can effectively avoid the background fluorescence of dissolved organic compounds in natural seawater. The probe Cy-DES has been successfully utilized to detect DES in complicated matrix such as seawater, shrimp and fish samples. In short, our probe Cy-DES owns great advantages and shows potential application in the DES detection in real samples.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.snb.2015.10.014.

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