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A bifunctional reagent regulated ratiometric electrochemiluminescence biosensor constructed on surfactant-assisted synthesis of TiO_2 mesocrystals for the sensing of deoxynivalenol

Hongli Zheng[a#] , Yumin Ke[b#], Huan Yi[b], Hong Dai[a*], Dandan Fang [a], Yanyu Lin[c] , Zhensheng Hong[a*], Xiuhua Li [a*]

a College of Chemistry and Material & College of Physics and Energy Fujian Normal University, Fuzhou, Fujian, 350108,China

b Fujian Provincial Maternity and Children`s Hospital, Fuzhou, Fujian, 350108,China

c Ministry of Education Key Laboratory of Analysis and Detection for Food Safety, and Department of Chemistry, Fuzhou University, Fuzhou 350002, P. R. China

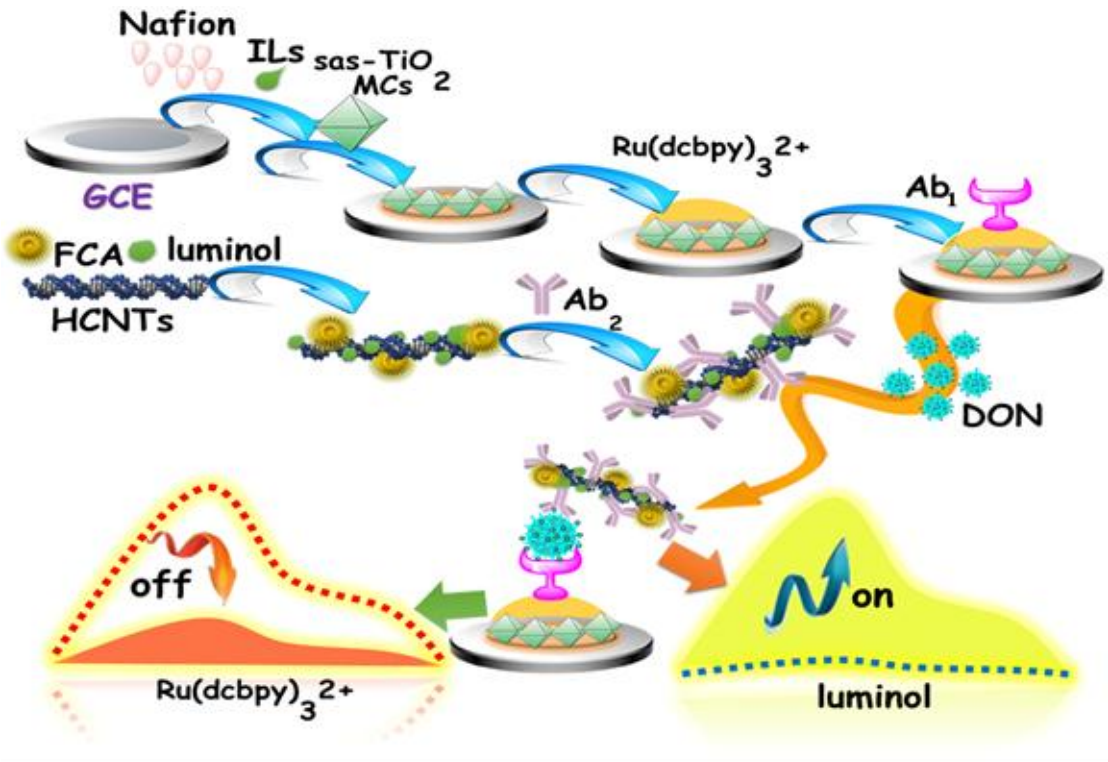
Abstract

Herein, a delicate bifunctional reagent regulated ratiometric electrochemiluminescence (ECL) biosensor was developed for the detection of deoxynivalenol (DON) by virtue of the ratio of two ECL signals from Luminol and tris(4,4′-dicarboxylicacid-2,2′-bipyridyl) ruthenium(II) dichloride ($Ru(dcbpy)_3^{2+}$).

Corresponding author: Fax: (+86)-591-22866135; E-mail: dhong@fjnu.edu.cn (H. Dai); # The author contributed to the work equally and should be regarded as co-first author.

Initially, surfactant-assisted synthesis of TiO_2 mesocrystals dispersing in Nafion and ionic liquid (IL) complex film held great promises in loading $Ru(dcbpy)_3^{2+}$ and amplifying the ECL signal. Additionally, helical carbon nanotube (HCNTs) with superior specific area and good conductivity emerging as a scaffold not only realized the high fixing of Luminol, ferrocenecarboxylic acid (FCA) and secondary antibody (Ab_2), but accelerated the electron transfer. It's noteworthy that FCA was first utilized as a bifunctional reagent to regulate the ratiometric ECL sensing mode on account of the influences in enhancing the ECL response of Luminol and quenching the ECL emission of $Ru(dcbpy)_3^{2+}$. On the basis of the sandwich-type immunoreactions between Ab_1, DON and Ab_2, an accurate and sensitive ratiometric ECL immunosensor was established for the detection of DON in a wide range from 0.05 pg/mL to 5 ng/mL with the detection limit of 1.67×10^{-2} pg/mL.

Graphical Abstract



Keywords:

Ratiometric ECL biosensor; Bifunctional reagent; Sas-TiO_2 MCs; Deoxynivalenol (DON)

1. Introduction

Worldwide, food safety has become a major issue and attracted extensive attention. [1, 2] Mycotoxins, a type of toxic secondary metabolites produced by fungi, are widely existed in wheat, barley, and corn with serious toxic effects. [3, 4] Among mycotoxins, deoxynivalenol (DON) has aroused the most attention due to its harmful influences on animals and humans. [5] It's a fact that DON is stable during cereal storage, processing and even heating, leading it to easily enter the human and animal

food chains and cause disease. [6,7] Therefore, it is of vital importance to construct sensitive and reliable analytical methods for the determination of trace DON.

Until now, analytical approaches such as high-performance liquid chromatography (HPLC), [8] liquid chromatography−mass spectrometry (LC−MS), [9] enzyme-linked immnosorbentassay method [ELISA], [10] chemiluminescent enzyme immunoassay [11] and electrochemical immunosensing method [12] were usually developed for the DON detection due to their high sensitivity and accuracy. However, some of these techniques require complex experimental process, expensive equipment costs and even a long reaction time, and some may suffer from the low selectivity or sensitivity. [13-15] Thus, it's urgent to develop a simple, rapid and sensitive method for the detection of DON in food.

Electrochemiluminescence (ECL), the combination of electrochemistry and chemiluminescence, has receiving considerable attention in biosensing fields owing to its intrinsic quality of high sensitivity, facile controllability and simplified optical equipment. [16, 17] Nevertheless, false positive or negative errors are inevitable from instrumental or environmental factors when the detection was depended on single signal change. [16, 18] To eliminate the interferences and make the detection results more believable, many efforts have been devoted to design various ratiometric ECL biosensor. Differ from conventional single ECL signal detection, the ratiometric ECL strategy requires a pair of luminophores. For example, a ratiometric ECL strategy for CEA based on CdS quantum dots as cathodic emitter and Luminol as anodic emitter

was developed by Huang et al. [19] Lei et al. demonstrated a ECL-RET system between PTC-NH_2 and $^1(O_2)_2^*$ to fabricate ratiometric aptasensor for Pb^{2+} detection. [20] In Fu's team, CdTe QDs and $Ru(dcbpy)_3^{2+}$ as new group of luminescent reagents were introduced into ratiometric ECL system to detect DA. [21] Nevertheless, the above ECL emission pairs exhibited imperfections more or less, such as quantum dots are generally bad biocompatibility and poisonous, and even require complicated preparation and functionalization process. Here, $Ru(dcbpy)_3^{2+}$ and Luminol as conventional ECL luminophors, were selected as new ECL emitter units due to the advantages of high ECL efficiency and good stability. Additionally, $Ru(dcbpy)_3^{2+}$ and Luminol intrinsic carboxyls or amidogens make them easy to directly bind with biomoleculars without any coupling agents, which greatly simplified experimental procedure. To improve the sensitivity of the ratiometric ECL biosensors, suitable quencher or enhancer has been introduced to regulate the ECL signals based on the biomimetic catalysis, ECL resonance energy transfer (ECL-RET) or surface plasmon resonance. Whereas, these strategies suffered from some disadvantages: (1) selecting suitable biomimetic catalyst is difficult and biomimetic catalysis requires long reaction time; (2) finding matched ECL energy donor and acceptor which possessed sufficient spectra overlap is hard; (3) the enhancer or quencher only presented single effect on one of ECL emitters in the ratiometric ECL sensors. Inspired by the above ideas, searching for a bifunctional reagent with both enhancing and quenching effects on ECL emitters in the ratiometric ECL biosensor is desirable. Ferrocenecarboxylic acid (FCA), a class of special organometallic compounds with high stability, unique

rigid and double-deck sandwiched structure has been revealed could effectively quench the ECL of $Ru(dcbpy)_3^{2+}$ and simultaneously enhance the ECL intensity of Luminol. [22, 23] However, few reports have focused on FCA as the bifunctional reagent in one ratiometric ECL pattern. Therefore, a new ECL report units including $Ru(dcbpy)_3^{2+}$ and Luminol was regulated by FCA to develop a ratiometric ECL biosensor.

Considering intramolecular ECL-RET from N-(aminobutyl)-N-(ethylisoluminol) (ABEI) to $Ru(bpy)_2(mcbpy)^{2+}$ may influence the construction of ratiometric ECL biosensor, two suitable scaffolds was indispensable to realize the respective immobilization of Luminol and $Ru(dcbpy)_3^{2+}$. Recently, TiO_2 mesocrystals have been extensively applied in biosensing field for its promising characteristics of tetrahedral structure, large specific area, high porosity and subunit alignment. [24, 25] However, the traditional synthesis methods often cause the heterogenous growth of nanocrystals. To conquer this drawback, introducing protecting agent in synthesis process is crucial to obtain stable and uniform distribution of mesocrystals. Generally, surfactant molecules play an important part in controlling the size and shape of the nanocrystals. Polyvinylpyrrolidone (PVP), a nontoxic polymer which has been reported to synthesis various structures of TiO_2 mesocrystals. [26, 27] Here, a surfactant-assisted synthesis of TiO_2 mesocrystals (sas-TiO_2 MCs) was prepared, which not only inherit all the merits of TiO_2 mesocrystals but exert improved properties of high crystallinity, high stability, especially the good photoelectric performance with the assistant of PVP. Besides, helical carbon nanotube (HCNTs), a kind of three-dimensional helical

structure carbon nanotube, showing charming properties of superior flexibility and mechanical stability, especially the intrinsic positive and negative curvatures on the surface, which distinctly enlarges the contact area with guest molecules. [28] Additionally, abundant reactive defect sites on HCNT would result in strong adsorption capability. [29] The abovementioned merits make HCNTs an excellent candidate for the immobilization of Luminol and FCA.

Hence, a new pair of ECL emitters of $Ru(dcbpy)_3^{2+}$ and Luminol was designed for ratiometric ECL sensing of DON for the first time. As illustrated in Scheme 1, Nafion-IL complex film incorporated with sas-TiO_2 MCs not only realized high loading of $Ru(dcbpy)_3^{2+}$ but accelerated electron-transfer, resulting in a strong and stable ECL emission. Moreover, HCNTs serving as another scaffold would greatly enlarge the capacity for immobilization of Luminol and other active molecules. Importantly, FCA as the bifunctional reagent, was first introduced into the system to regulate the ratio of two ECL signals based on the enhancement in ECL of Luminol and the quenching in that of $Ru(dcbpy)_3^{2+}$, which effectively improve the sensitivity of the ratiometric ECL biosensor. In a word, the ratiometric ECL sensing methodology realized the accurate and sensitive detection of DON and provided great promise in food safety detection and clinical diagnosis.

Scheme 1

2. Experimental Section

2.1. Materials and reagents

Tris (4,4′-dicarboxylicacid-2,2′-bipyridyl) ruthenium(II) dichloride

($Ru(dcbpy)_3^{2+}$) was received from Sahn Chemical Technology Co. (Shanghai, China). Luminol and 5% Nafion was purchased from Sigma-Aldrich. Ferrocenecarboxylic acid (FCA, 97%) was obtained from International Laboratory USA. 1-Butylpyridinium tetrafluoroborate (Ionic liquid, 10 mg/mL) was purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA, 96–99%), N-hydroxy succinimide (NHS) and 1-ethyl-3-(3-di-methylaminopropyl) carbodiimide hydrochloride (EDC) were obtained from Shanghai Medpep Co. (Shanghai, China). Deoxynivalenol (DON) standard solutions, DON antibody and zearalenone (ZEA) standard solutions were purchased from Wuxi Biosensor Technology Co. (Jiangsu, China). Aflatoxin B_1 (AFB_1) standard solutions were purchased from Jiangsu Beosen Food Safety Technology Co. (Jiangsu, China). Prostate specific antigen (PSA) standard solutions were purchased from Linc-Bio Science Co. (Shanghai, China). Phosphate buffer solution (PBS) with different pH values were prepared by mixing 0.2 M Na_2HPO_4 and 0.3 M NaH_2PO_4, and adjusted with 0.1 M HCl or NaOH under monitoring of PHS-3C exact digital pH metre (Shanghai, China). Sas-TiO_2 MCs (2 mg/mL) and HCNTs (5 mg/mL) were prepared in this work.

2.2. Synthesis of sas-TiO_2 MCs

Sas-TiO_2 MCs was synthesized as following. Simply, 1.0 g of polyvinylpyrrolidone (PVP) and 4.0 g of sodium dodecyl sulfate (SDS) was successively dissolved into 100 mL 2.1 M HNO_3 solution under sonication. After that, 2.0 mL titanium(IV) isopropoxide (TIP) was poured into the above mixture solution

and kept at 70 °C for 48 h under rigorously stirring. Finally, the sas-TiO_2 MCs powder was obtained by centrifugation, washed and dried at 60 °C overnight.

2.3. Preparation of HCNTs and functinalized HCNTs

The HCNTs was synthesized as follows. Firstly, a quartz plate was sonicated in acetone for 30 min, then rinsed by deionized water. Afterwards, it was treated with 0.1 M iron nitrate ($Fe(NO_3)\cdot 9H_2O$) solution and dried at room temperature. Subsequently, the above quartz plate was placed in the middle of horizontal quartz tubular furnace and kept at 800 ◦C under H_2 atmosphere for 90 min. After that, CNTs were grown on the quartz plate by injecting ethanol into the furnace. The resultant HCNTs was acquired after the samples naturally cooling down to room temperature under N_2 atmosphere. The carboxylic group-functionalized HCNTs was further prepared according to previous work with a minor change. [31] Initially, 5 mg HCNTs was dispersed into 20 mL H_2SO_4/HNO_3 (V:V=3:1) solution and sonicated at 40 ºC for 3 h. Then the mixture was cooled to room temperature and diluted to 5 mL with ultrapure water. Subsequently, the product was prepared by filtrating with a nylon membrane (0.22 μm) and dried in a vacuum oven at 60 ºC. Finally, the carboxylic group-functionalized HCNTs was re-dispersed into ultrapure water with the concentration of 5 mg/mL.

2.4. Preparation of Nafion-IL-sas-TiO_2 MCs composites

Simply, 80 μL of 0.5% Nafion and 20 μL of 10 mg/mL IL was mixed under room temperature. After stirring for a few minutes, 100 μL 2 mg/mL sas-TiO_2 MCs was poured into the above solution under stirring. Finally, the Nafion-IL-sas-TiO_2 MCs

complex film was formed after mixing for 30 min at room temperature and stored in the refrigerator at 4 ºC for use.

2.5. Preparation of HCNTs-Luminol@FCA-Ab$_2$ Hybrids

To prepare HCNTs-Luminol complex, 20 μL EDC/NHS (20 mM:5 mM) mixed at equal volume was added into 100 μL 5 mg/mL HCNTs to activate the carboxyl. After 1 h, 100 μL of 10 mM Luminol was poured into the above solution and reacted for 4 h to immobilize Luminol via amide reaction. Finally, the composites were obtained by centrifugation and washing to remove the unconjugated Luminol and then re-dispersed in ultrapure water with a concentration of 5 mg/mL for next use. The successful preparation of HCNTs-Luminol complex was confirmed by the UV-vis absorption spectra (Fig. S1A).

Similarly, the FCA-Ab$_2$ biocomposite was synthesized via the amide reaction between the carboxyl from FCA and the amino group of Ab$_2$. Simply, 20 μL EDC/NHS mixed at equal volume (20 mM:5 mM) was poured into 100 μL of 10 mM FCA to activate the carboxyl of FCA under stirring, followed by adding 30 μL of 10 mg/mL Ab$_2$ solution with stirring overnight at 4 ºC. After the further purification of FCA-Ab$_2$ biocomposite through centrifuging and washing with ultrapure water, it was stored at 4 ºC for further use. And the UV-vis absorption spectra was performed to characterize the formation of FCA-Ab$_2$ biocomposite (Fig. S1B).

The final HCNTs-Luminol@FCA-Ab$_2$ hybrids were prepared though the electrostatic adsorption between positively charged of HCNTs-Luminol composites and negatively charged Ab$_2$-FCA complex. Specifically, 100 μL the above prepared

FCA-Ab_2 composite was added into 100 μL 5 mg/mL HCNTs-Luminol with stirring for 3 h at room temperature. Then, 30 μL bovine serum albumin (BSA, 1%) was used to block the nonspecific binding sites. Centrifuging and washing by ultrapure water, the HCNTs-Luminol@FCA-Ab_2 hybrids were obtained. And the UV-vis absorption spectra was displayed in Fig. S1C to confirm the formation of HCNTs-Luminol@FCA-Ab_2 hybrids.

2.6. Fabrication of the ratiometric ECL biosensor

Firstly, the bare glassy carbon electrode (GCE, 3 mm in diameter) was polished to a mirror with 0.3 and 0.05 μm alumina slurry followed by rinsing thoroughly with alcohol and water. After drying, 5 μL of Nafion-IL-sas-TiO_2 MCs solution was casted on the clean GCE surface and dried under an infrared lamp. Subsequently, the modified GCE was immersed into 1.0×10^{-3} mol/L $Ru(dcbpy)_3^{2+}$ for 1 h to finish the immobilization of $Ru(dcbpy)_3^{2+}$. After rinsing with ultrapure water and dried under an infrared lamp, 5 μL of EDC/NHS (molar ratio 4:1) was spread onto the modified electrode to activate the carboxyl of $Ru(dcbpy)_3^{2+}$ for 30 min at 4 °C. Then, 5 μL of Ab_1 (1 mg/mL) was integrated into the modified GCE though the amide reaction between carboxyl from $Ru(dcbpy)_3^{2+}$ and amino from Ab_1 and incubated for 40 min at 4 °C. After removing the excess Ab_1 with ultrapure water, 5 μL of BSA (1%) was moved on the electrode to block nonspecific binding sites for 30 min at 4 °C. Afterwards, the modified electrode was incubated with 5 μL different concentrations of DON standards for 40 min at 4 °C. Finally, 5 μL of HCNTs-Luminol@FCA-Ab_2 hybrids were introduced into the electrode via the specific recognition between Ab_2

and DON. After reacting for 40 min at 4 °C and washing by ultrapure water, the ratiometric ECL biosensor was prepared to be measured.

Remarkably, the above each modification process must guarantee the electrode surface uniformity and neatness, which was crucial in keeping the stable ECL signal. After every decoration, the electrode surface must wash with ultrapure water to remove the residues, which makes the analysis more quantitative. Moreover, when the electrode was immersed into solution to finish the adsorption, the solution should be stirred to assure the homogeneous adsorption. [32,33]

3. Results and discussion

3.1. Characterization of materials.

The typical scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were applied for the morphological and structural characterization of the sas-TiO_2 MCs. Seen from Fig. 1A, numerous monodisperse nanoparticles with the average size of 40−60 nm were formed. The TEM image in Fig. 1B showed the rough surface and porous structure of those nanoparticles. Moreover, numberous tiny subunits were observed in one nanoparticle, which confirmed the high crystallinity of sas-TiO_2 MCs. Fig. 1C presented the X-ray diffraction (XRD) patterns of sas-TiO_2 MCs, the main diffraction peaks at 25.28, 37.93, and 48.38 degree were indexed to (101), (004), and (200) crystal planes of anatase TiO_2, respectively. Additionally, the broadened diffraction peaks domenstrate a small crystallite size of the samples. Based on the (101) diffraction peak, the smaller average crystallite size was

calculated to about 10 nm according to the Scherer equation. [34] All the obove characterization suggested the sas-TiO_2 MCs was synthesised successfully.

The morphology of HCNTs was characterized by scanning electron microscopy (SEM). As exhibited in Fig. 1D, large amounts of well-defined and ordered helical-like structure with the length of 10–40 μm were observed, demonstrating the successful formation of HCNTs and the nature of larger specific area of HCNTs.

Fig. 1

3.2. Characterization of ratiometric ECL biosensor

Electrochemical impedance spectroscopy (EIS) serving as a typical tool reflecting the electron-transfer resistance as employed to characterize the interface properties of modified electrodes. [35-37] As displayed in Fig. 2A, the bare GCE showed a small electron-transfer resistance (R_{et} = 856 Ω, curve a), while an increased impedance value of 2035 Ω was obtained at the Nafion-IL-sas-TiO_2 MCs/$Ru(dcbpy)_3^{2+}$ modified GCE (curve b) because of the extremely poor conductivity of Nafion hindered the electron transmission, though the ionic liquid (IL) could improve the conductivity of Nafion in some extent. Afterwards, the impedance values gradually increased with the successively modified of Ab_1 (R_{et} = 3247 Ω, curve c), DON (R_{et} = 6065 Ω, curve d) and Ab_2-bioconjugates (R_{et} = 11319 Ω, curve e), which was resulted from the insulation of the protein. The above results confirmed the successful assembly of the modified electrode.

To further prove the successful construction of the immunosensor, ECL intensity

at each modification step was recorded in Fig. 2B. It is clear that no ECL signal was detected at bare GCE (curve a), while strong ECL emission of 12395 a.u was present at about +1.1V after the electrode was modified by Nafion-IL-sas-TiO_2 MCs/$Ru(dcbpy)_3^{2+}$ (curve b), which was from the luminophore of the $Ru(dcbpy)_3^{2+}$. With the incubation of Ab_1 (curve c) and DON (curve d), the ECL intensity presented consecutively declined tendency from 5650 a.u to 3181 a.u due to the blocked electron-transfer of protein. However, when HCNTs-Luminol@FCA-Ab_2 was grafted to the surface of electrode, the ECL intensity at +1.1 V dramatically decreased to 2185 a.u and another new anode ECL peak of 4652 a.u appeared at about +0.5 V (curve e), corresponding to the ECL peak of Luminol, which was further proved by the cyclic voltammetry (CV) curve in Fig. S2. The above results certified the successful construction of ratiometric ECL biosensor.

To further demonstrate the "off-on" process in the preparation of the ratiometric ECL biosensor, the ECL-potential responses at different modified electrodes was investigated in Fig. 2C. Obviously, the Nafion-IL-sas-TiO_2 MCs/$Ru(dcbpy)_3^{2+}$ modified electrode received one strong ECL emission (curve a), confirming the high loading of $Ru(dcbpy)_3^{2+}$; while a declined ECL intensity was observed when Ab_1 and DON was assembled to the electrode (curve b), which was attributed to large protein molecular of Ab_1 and DON impeded the electron transfer. Interestingly, after the incubation of HCNTs-Luminol@FCA-Ab_2, the ECL emission of $Ru(dcbpy)_3^{2+}$ decreased again and another ECL emission peak from Luminol was appeared (curve c). The above "off-on" phenomenon was mainly regulated by the bifunctional reagent

of FCA, which could enhance the ECL of Luminol and quench the ECL from $Ru(dcbpy)_3^{2+}$. Fig. 2D showed the logic gate system, which defined the ECL signal of $Ru(dcbpy)_3^{2+}$ and Luminol as input and the ratio of two signal as output. As seen from the table, output of the logic gate occurs only if when both inputs are present, indicating two luminophors generated ECL emission at the same time was the necessary condition of ratiometic ECL biosensor.

Fig. 2

To reveal the surperiority of the matrix of Nafion-IL-sas-TiO_2 MCs hybrids, the ECL-time profiles and corresponding cyclic voltammogram curves of different modified electrodes was recorded in Fig. 3A. It's worth noting that Nafion-IL/$Ru(dcbpy)_3^{2+}$ modified electrode generated a higher ECL signal and a set of more apparent peaks current at +1.1 V (curve b) than that of Nafion/$Ru(dcbpy)_3^{2+}$ (curve a), which due to the IL could improve the conductivity of Nafion and accelerate the electron transfer. Miraculously, when Nafion-IL-sas-TiO_2 MCs hybrids as the carrier to load $Ru(dcbpy)_3^{2+}$, a greatly enhanced ECL emission and peak current was obtained (curve c), which was ascribed to the following aspects: (1) The excellent properties of high porosity, large surface area enabled sas-TiO_2 MCs to load high quantity of $Ru(dcbpy)_3^{2+}$; (2) sas-TiO_2 MCs obtained the improved crystallinity and photoelectric performance with the assistance of PVP surfactant and thus acted as a direct electron relay, which accelerated the electron-transfer rate from the reduced $Ru(dcbpy)_3^{2+}$ species to the conduction band (CB) of sas-TiO_2 MCs and then from the the CB of sas-TiO_2 MCs to the oxidized $Ru(dcbpy)_3^{2+}$ species, resulting in an apparent

amplification of ECL intensity [30]; (3) hydrophobic cation of $Ru(dcbpy)_3^{2+}$ could be easily incorporated into the composite films consists of cation-exchangeable Nafion and sas-TiO_2 MCs via both ion-exchange process and hydrophobic interactions, resulting in an increase of uptake rate of $Ru(dcbpy)_3^{2+}$. The above points not only revealed the successful construction of the matrix but proved the advisable selecting of Nafion-IL-sas-TiO_2 MCs hybrids as the sensing platform.

Meanwhile, different probes modified electrodes were compared via the ECL intensity and peak current conducted in PBS (pH 8.0). As displayed in Fig. 3B, the ECL signal and peak current was invisible at HCNTs modified electrode (curve a), while an obvious ECL emission and a pair of peaks current at + 0.5 V was received at HCNTs-Luminol modified electrode (curve b), indicating the ECL resource was from Luminol. As expected, a higher ECL signal and two sets of peaks current at + 0.5 V and + 0.3 V corresponding to Luminol and FCA, respectively, was observed after the modification of HCNTs-Luminol@FCA-Ab_2, revealing the FCA-Ab_2 was successfully absorbed into the HCNTs-Luminol and FCA could boost the ECL emission of Luminol.

Fig. 3

3.3 The regulated function of FCA on the ratiometric ECL mode

In order to demonstrate the effects of FCA on the ratiometric ECL biosensor, the ECL intensity of $Ru(dcbpy)_3^{2+}$ and Luminol was investigated under different concentrations of FCA from 0 to 0.2 mM. As exhibited in Fig. S3A, the ECL intensity of $Ru(dcbpy)_3^{2+}$ declined with the increasing concentrations of FCA from 0 to 0.2 mM

(curve a to d), revealing FCA could effectively quench the ECL response of $Ru(dcbpy)_3^{2+}$. The quenching mechanism was considered to be the electron transfer between excited-state $Ru(dcbpy)_3^{2+}*$ and FCA^+. Firstly, the FCA was oxidized to produce FCA^+, which further reacts with the excited-state $Ru(dcbpy)_3^{2+}*$, in other word, the FCA^+ can inhibit generation of $Ru(bpy)_3^{2+}*$ and thus weakened the ECL emission of $Ru(dcbpy)_3^{2+}$. [22] On the contrary, the ECL intensity of Luminol was elevated with the rising amounts of FCA (curve a to d), as shown in Fig. S3B, clarifying the enhanced effect of FCA on the ECL of Luminol. The mechanism was possible that oxidized FCA (FCA^+) could catalyze the generation of reactive oxygen species ($OH^{\bullet}$, $O_2^{\bullet-}$) and further promote the oxidization of Luminol. [38] The specific reaction process was as follows:

$$FCA - e^- \rightarrow FCA^+ \quad (1)$$

$$Ru(bpy)_3^{2+} - e^- \rightarrow Ru(bpy)_3^{3+} \quad (2)$$

$$Ru(bpy)_3^{3+} \rightarrow Ru(bpy)_3^{2+*} \quad (3)$$

$$Ru(bpy)_3^{2+*} \rightarrow Ru(bpy)_3^{2+} + hv \quad (4)$$

$$FCA^+ + Ru(bpy)_3^{2+*} \rightarrow Ru(bpy)_3^{3+} + FCA \quad (5)$$

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \quad (6)$$

$$H_2O_2 \xrightarrow{FCA^+} OH^{\bullet} + O_2^{\bullet-} \quad (7)$$

$$Luminol - e^- \xrightarrow{-2H^+} Luminol^{\bullet-} \quad (8)$$

$$Luminol^{\bullet-} + OH^{\bullet} / O_2^{\bullet-} \rightarrow (Luminol_{OX})^* + products \quad (9)$$

$$(Luminol_{OX})^* \rightarrow Luminol + hv \quad (10)$$

The above results attested to the fact that FCA could be utilized as bifunctional reagent to regulate the ECL ratiometric mode. By virtue of the double effects of FCA in quenching the ECL of $Ru(dcbpy)_3^{2+}$ and enhancing the luminous efficiency of Luminol, a highly sensitive and accurate ratiometric ECL biosensor was realized.

3.4. Optimization of experiment conditions

To obtain satisfactory performance in the ECL assay, several conditions including the volume ratio of IL and Nafion, the concentration of sas-TiO_2 MCs and the adsorption time of $Ru(dcbpy)_3^{2+}$ on Nafion-IL-sas-TiO_2 MCs were optimized. As shown in Fig. 4A, a best ECL signal was received at 20% volume ratio of 10 mg/mL IL and 0.5% Nafion. The optimal concentration of sas-TiO_2 MCs was 2.0 mg/mL, at which receiving a higher ECL intensity as investigated in Fig. 4B. Moreover, the adsorption time of $Ru(dcbpy)_3^{2+}$ on Nafion-IL-sas-TiO_2 MCs was researched in Fig. 4C, the ECL signal elevated from 30 to 60 min and reached a relatively stable value at 60 min. Therefore, 60 min was selected as the optimum adsorption time of $Ru(dcbpy)_3^{2+}$ on Nafion-IL-sas-TiO_2 MCs. Additionally, several experimental parameters of the bioprobe such as the immobilized time of Luminol on HCNTs and the adsorption time of FCA-Ab_2 on HCNTs-Luminol were evaluated in Fig. S4, the ideal immobilized time for Luminol was 4 h and the optimal adsorption time of FCA-Ab_2 on HCNTs-Luminol was 3 h. Meanwhile, the incubation time of Ab_1, DON, HCNTs-Luminol@FCA-Ab_2 composites and the pH value of the testing solution were investigated (Fig. S5). The optimal incubation time of Ab_1, DON, HCNTs-Luminol@FCA-Ab_2 composites was 40 min and the optimal pH value of the testing solution was 8.0. The detailed explanation was submitted in supporting information.

Fig. 4

3.5. Analytical performance of the immunosensor

Under the optimal conditions, different DON concentrations were tested with the designed ECL immunosensor. From Fig. 5A, the ECL intensity of Luminol increased, while the ECL intensity of $Ru(dcbpy)_3^{2+}$ decreased with the elevated concentration of DON from 0.05 pg/mL to 5 ng/mL, resulting in a ratiometric determination of DON. Fig. 5B presented a good linear relationship between the logarithm concentration of DON and the ECL intensity ratio of Luminol to $Ru(dcbpy)_3^{2+}$. The regression equation was I= 2.288+0.48logC (ng mL^{-1}) with a determination coefficient square of R^2=0.992 (where I was the ECL intensity ratio of Luminol to $Ru(dcbpy)_3^{2+}$ and C was the concentration of DON). In addition, a good linear relationship between the logarithm concentration of DON and the ECL intensity of Luminol and $Ru(dcbpy)_3^{2+}$ was described in Fig. S6. The determination coefficient was calculated to be 0.996 and 0.992, respectively. It's noteworthy that the limit of detection (LOD) and limit of quantitation (LOQ) was calculated to be 1.67×10^{-2} pg/mL and 5.0×10^{-2} pg/mL according to related reports [39], which was lower than other methods displayed in Table S1 for DON detection, indicating the excellent analytical performance of the ratiometric immunosensor for the determination of DON.

3.6. Selectivity and stability of the proposed ECL immunosensor

To check the selectivity of the immunosensor, the interference experiments were performed using 500 pg/mL of zearalenone (ZEN), prostate specific antigen (PSA) and aflatoxin B_1 (AFB_1). As shown in Fig. 5C, above interferences caused no

significant ECL intensity changes, while in the case of DON, the ECL intensity ratio ($ECL_{Luminol}/ECL_{Ru(dcbpy)32+}$) increased instantly, which confirmed the high selectivity towards DON. Additionally, the stability of the ECL ratiometric immunosensor was evaluated under consecutive cyclic potential scans for fifteen cycles at 500 pg/mL of DON. As presented in Fig. 5D, the relative standard deviation (RSD) was 0.73% and 1.33%, corresponding to Luminol and $Ru(dcbpy)_3^{2+}$, respectively, indicating acceptable stability of the immunosensor.

Fig. 5

3.7. Application of the immunosensor in practical samples

To validate the feasibility of the ECL strategy, the recovery experiments were performed by a standard addition method with sauce as model. Before assay, the sauce sample was diluted 5 times, then standard DON with different concentrations was spiked in sauce sample. With the proposed ratiometric ECL immunosensor, the DON of founded was measured. As shown in Table 1, the recoveries of DON were ranged from 92.3~98.5% and the RSD was 2.3~3.2% for DON, illustrating the great potential of the prepared ECL ratiometric strategy in real sample analysis.

4. Conclusions

In summary, a bifunctional reagent regulated ratiometric ECL biosensor for the sensing of DON was developed based on $Ru(dcbpy)_3^{2+}$ and Luminol as new ECL emitter units, which was brought to light for the first time. In this protocol, sas-TiO_2 MCs with excellent properties not only served as a matrix to hold large amounts of $Ru(dcbpy)_3^{2+}$ but acted as the electron relay to accelerate the electron transfer from

the reduced $Ru(dcbpy)_3^{2+}$ species to the oxidized $Ru(dcbpy)_3^{2+}$ species, which significantly facilitated the ECL response of $Ru(dcbpy)_3^{2+}$. Additionally, HCNTs, as the bioprobe scaffold, achieved the high loading of Luminol and other active molecules owing to its extensive contact area and strong adsorption capability. What's important, FCA was first used as the bifunctional reagent in ratiometric ECL strategy to regulate the "off-on" ratiometric mode in view of the enhancement in the ECL response from Luminol and quenching in the ECL from $Ru(dcbpy)_3^{2+}$, which avoided the addition of multiple coreactants and simplified experimental process. Therefore, on the basis of the bifunctional reagent regulated pattern, the ratiometric ECL strategy provided an accurate and sensitive method for the determination of DON with lower detection limit and wider linear range, thus holding promising perspective for the food safety detection and clinical diagnosis .

Acknowledgment

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Figure captions

Scheme 1. The fabrication process of the ratiometric ECL immunosensor.

Fig. 1 (A) SEM image, (B) TEM image and (C) XRD pattern of sas-TiO_2 MCs. (D) SEM image of HCNTs.

Fig. 2 (A) EIS curves and (B) ECL responses of (a) GCE, (b) Nafion-IL-sas-TiO_2 MCs/$Ru(dcbpy)_3^{2+}$/GCE, (c) Nafion-IL-sas-TiO_2 MCs/$Ru(dcbpy)_3^{2+}$/Ab_1/GCE, (d) Nafion-IL-sas-TiO_2 MCs/$Ru(dcbpy)_3^{2+}$/Ab_1/DON/GCE, (e) Nafion-IL-sas-TiO_2 MCs/$Ru(dcbpy)_3^{2+}$/Ab_1/DON/Ab_2-FCA@Luminol-HCNTs/GCE. (C) ECL responses of different electrodes of (a) Nafion-IL-sas-TiO_2 MCs/$Ru(dcbpy)_3^{2+}$/GCE, (b) Nafion-IL-sas-TiO_2 MCs/$Ru(dcbpy)_3^{2+}$/Ab_1/DON/GCE, (c) Nafion-IL-sas-TiO_2 MCs/$Ru(dcbpy)_3^{2+}$/Ab_1/DON/Ab_2-FCA@Luminol-HCNTs/GCE (Note: graph of C is a part of B) in 0.1 M PBS (pH = 8.0). (D) Refers to the logic gate system.

Fig. 3 (A) ECL − time profiles of different modified electrodes (a) Nafion/$Ru(dcbpy)_3^{2+}$/GCE, (b) Nafion-IL/$Ru(dcbpy)_3^{2+}$/GCE, (c) Nafion-IL-sas-TiO_2 MCs/$Ru(dcbpy)_3^{2+}$/GCE. (B) ECL − time profiles of (a) HCNTs/GCE, (b) HCNTs@Luminol/GCE, (c) HCNTs-Luminol@FCA-Ab_2/GCE. Insets of (A) and (B) were the corresponding cyclic voltammogram curves in 0.1 M PBS (pH = 8.0).

Fig. 4 Effects of (A) the volume ratio of Nafion and IL, (B) the concentration of sas-TiO_2 MCs and (C) the adsorption time of $Ru(dcbpy)_3^{2+}$ on Nafion-IL-sas-TiO_2 MCs for the ECL responses of immunosensor in 0.1 M PBS (pH =8.0).

Fig. 5 (A) ECL responses of immunosensor and (B) Calibration plot of the ECL intensity with different concentrations of DON from 50 fg/ml to 5 ng/ml in 0.10 M

PBS (pH = 8.0). (C) The selective of the proposed ECL immunosensor and (D) the stability of the proposed ECL immunosensor under consecutive cyclic potential scans for 15 cycles.

Scheme 1

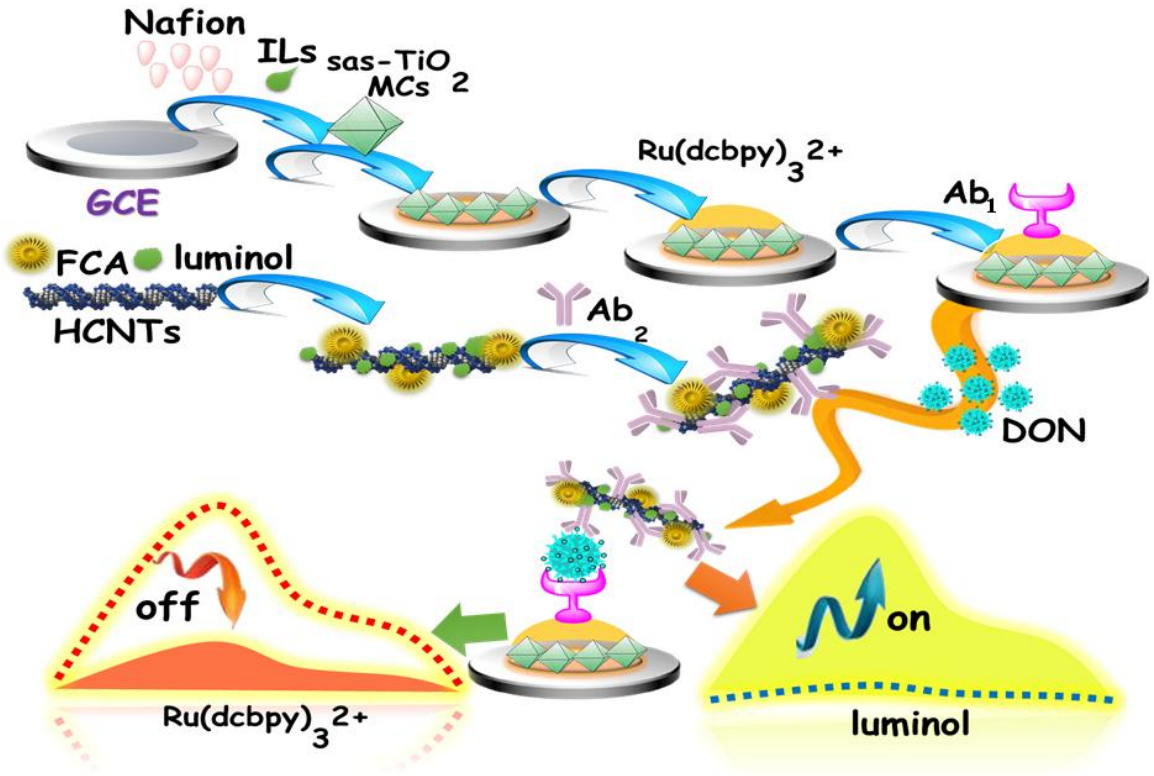


Figure 1

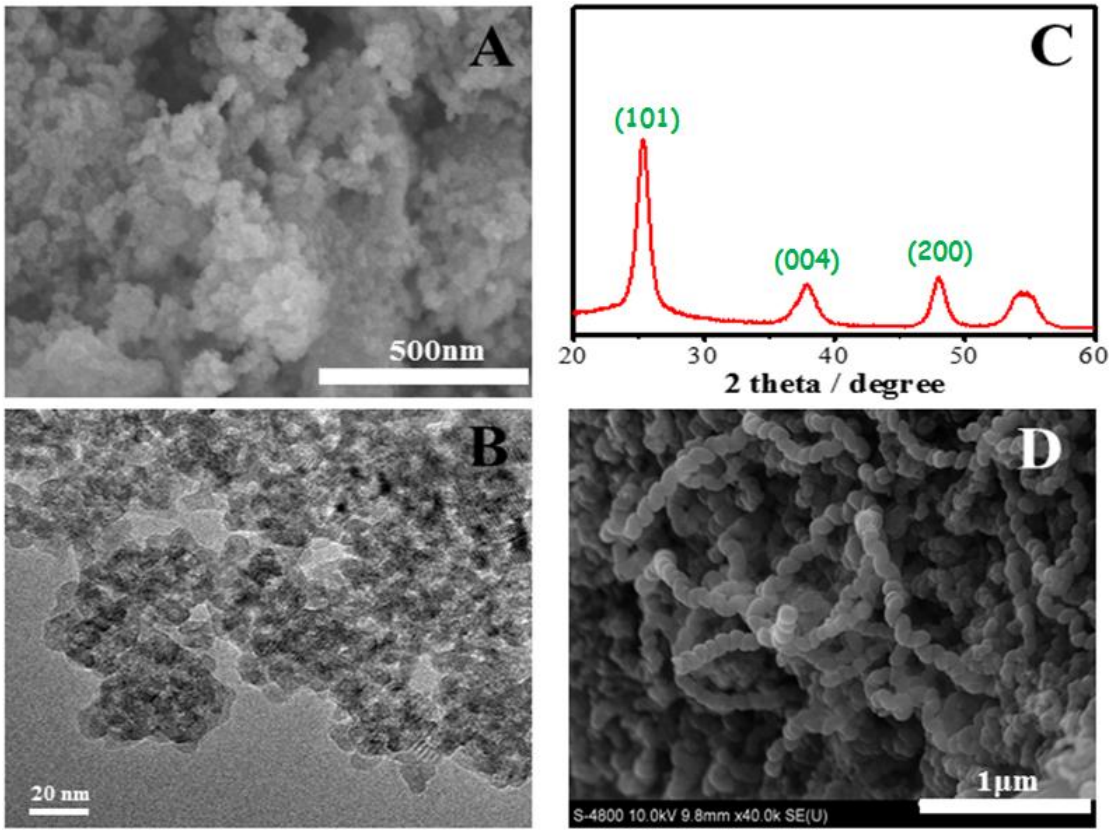
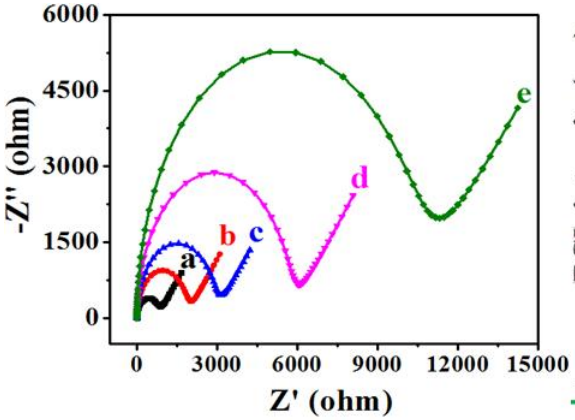
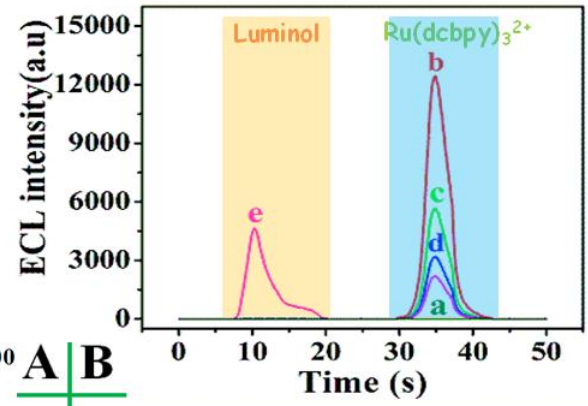
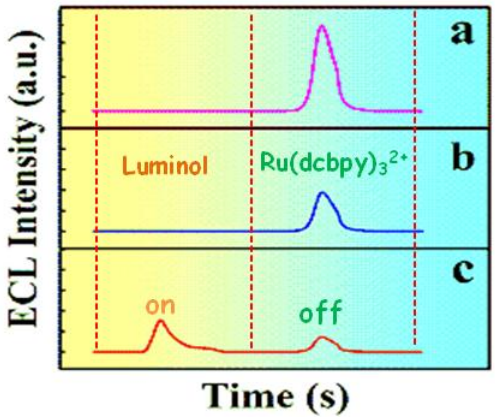


Figure 2







A | B
C | D

Inputs		Outputs
$Ru(dcbpy)_3^{2+}$	Luminol	Ratio of two ECL signals
0	0	0
1	0	0
0	1	0
1	1	1

Figure 3

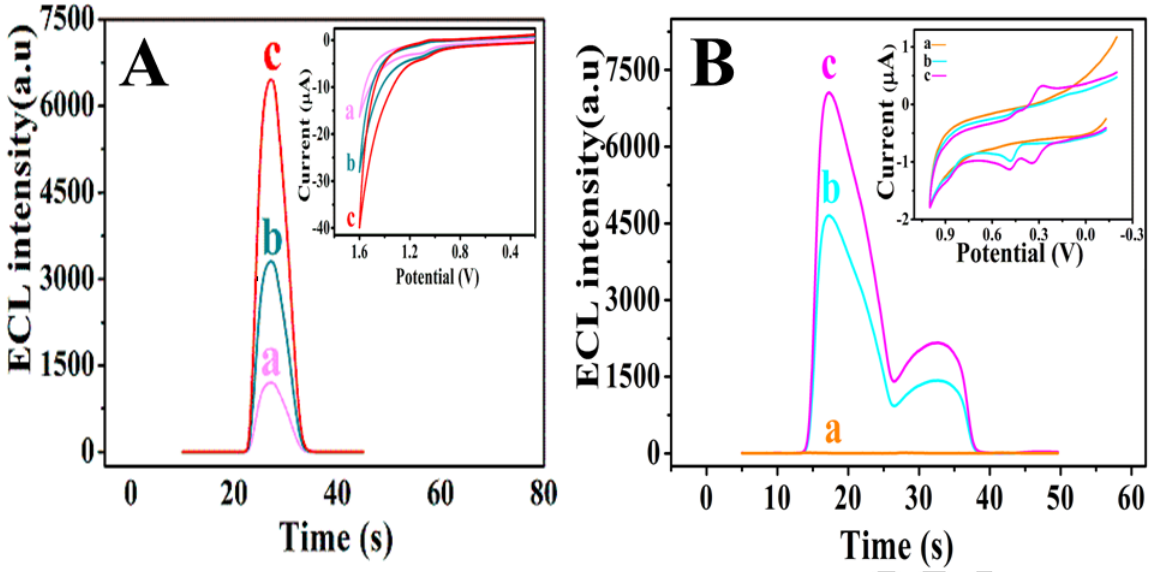


Figure 4

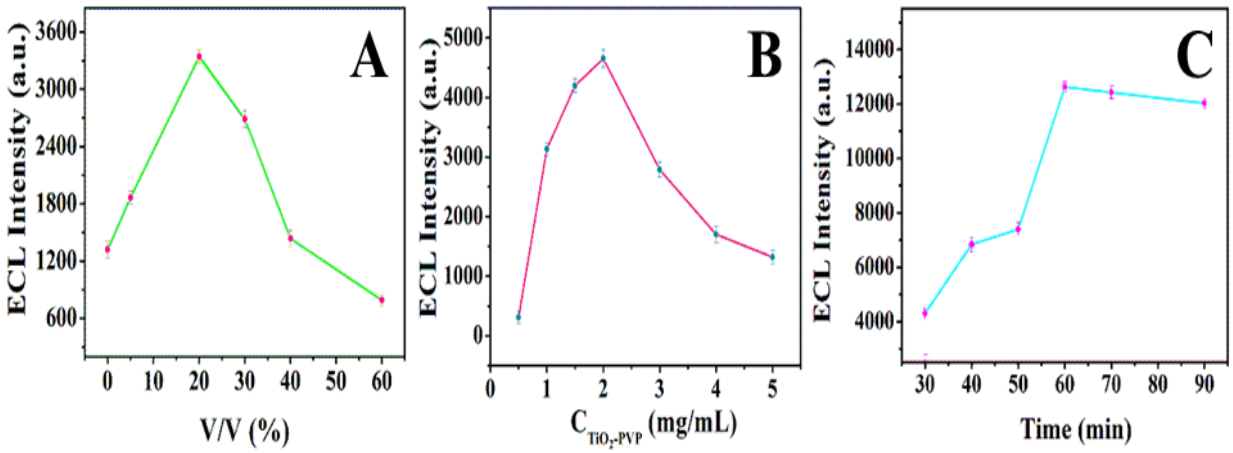


Figure 5

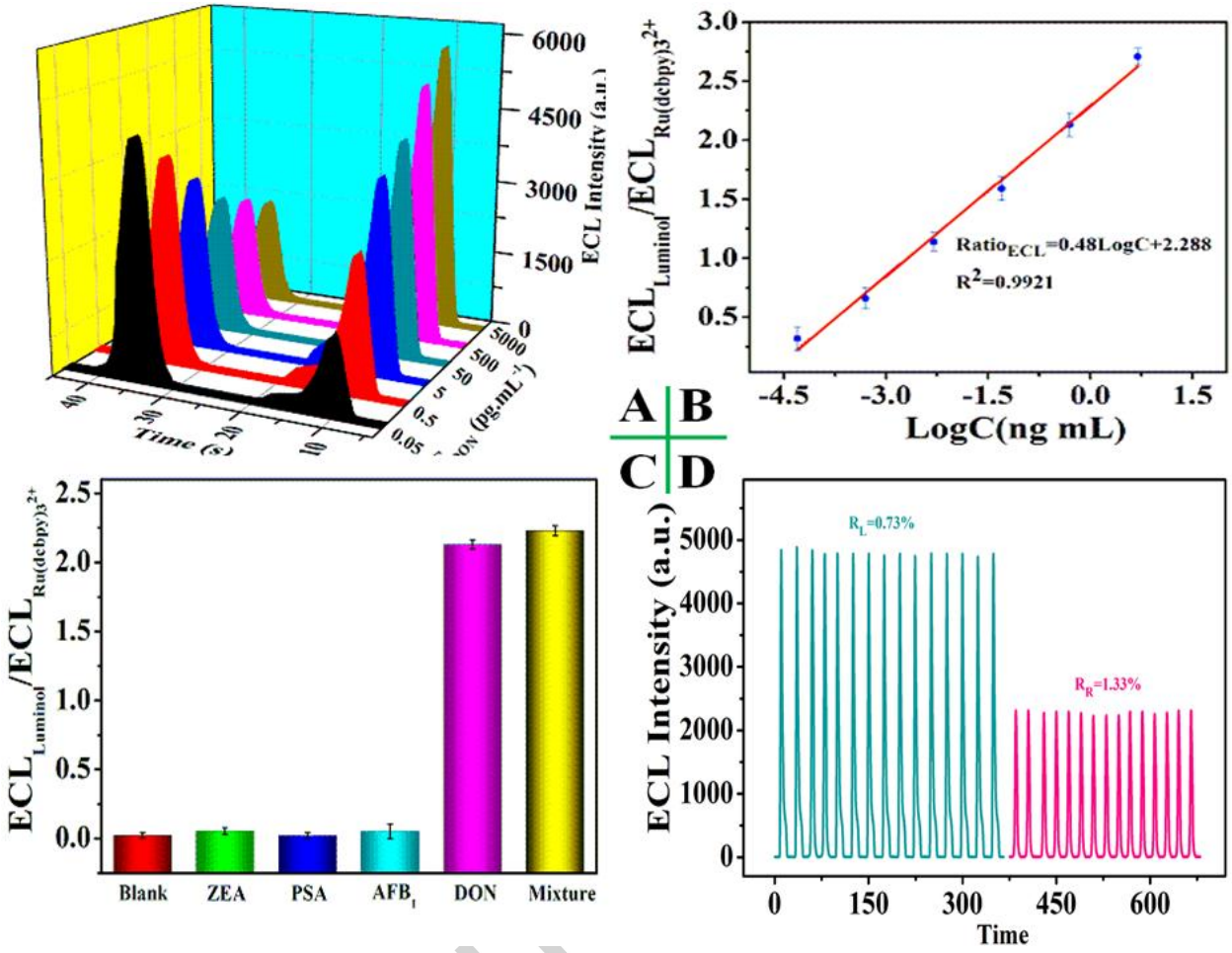


Table 1 Recoveries tests of DON in real samples (n=3) [a].

Samples	DON (pg/mL)	Added (pg/mL)	Found (pg/mL)	Recovery (%)	RSD (%)
Sauce	0.0295	5	4.65	92.3	3.2
		50	49.3	98.5	2.8
		500	472	94.3	2.3

[a] n is the repetitive measurements number.

Highlights

- A ratiometric ECL biosensor was designed based on Luminol and Ru $(dcbpy)_3^{2+}$ as ECL emitters units for DON detection.
- The surfactant-assisted synthesis of TiO_2 mesocrystals and HCNTs were employed as biosensing platform to amplify the ECL signal of two kinds of luminophore.
- The bifunctional reagent regulation mechanism in quenching the ECL intensity of $Ru(dcbpy)_3^{2+}$ and enhancing the ECL signal of Luminol improve the sensitivity of biosensor.