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Direct and indirect fluorescent detection of tetracyclines using dually emitting carbon dots

Fei Qu[1,2] · Zhe Sun[1,2] · Dongya Liu[1,2] · Xianen Zhao[1,2] · Jinmao You[1,2,3]

Abstract The authors describe dual-emission carbon nanodots containing blue emitters (BE; peak emission at 385nm under 315 nm excitation) and yellow emitters (YE; peak emission at 530 nm under 365nm excitation), and how they can be applied to direct and indirect determination of tetracyclines (TCs). The direct detection scheme is based on the finding that tetracycline (TET), oxytetracycline, chlortetracycline and doxycycline quench the two emissions of the carbon dots. While direct determination is rapid and convenient, it cannot differentiate between TCs. The indirect detection scheme, in contrast, is based on the finding that Al (III) ions enhance the fluorescence of the YE in the carbon dots, and that they cause a blue shift in emission. It is, however, known that TET forms a strong complex with Al (III), and this can inhibit the interaction between Al (III) and the YE, so that the fluorescence of YE is not enhanced and blue-shifted by Al (III) in the presence of TET. This finding is exploited in a fluorescence turn-on/off assay for TET that can distinguish TET from other TCs. The linear range of indirect determination for TET extends from 1 nM to 30 μM, and the limit of detection is 0.52 nM. The indirect method was successfully applied to the determination of TET in spiked milk, fish and pork, and recoveries ranged from 91.7 to 102 %.

Keywords Tetracycline · Oxytetracycline · Chlortetracycline · Doxycycline · Fluorometry · Quenching

Introduction

Tetracycline (TET) is a member of tetracyclines (TCs) antibiotics family, which also includes oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DOX). In the late 1940s, TCs were widely used for treatment of infectious diseases in humans [1]. However, long-term and repeated intake of TCs are claimed to have serious side effects, such as yellowing of teeth, anaphylactic reaction, gastrointestinal disturbance and hepatotoxicity [2]. Therefore, so far, TCs are usually employed in veterinary medicine for the treatment of animal infections and animal growth promoter rather than humans [3], because of their broad-spectrum antimicrobial, good oral absorption, and low cost [4]. However, some farmers overcommit TCs in animal feeding to pursue the interests, causing accumulation of antibiotics in milk, meat, honey, fish and eggs [5–8]. Intake of the food with antibiotics residues in long-time, in fact, a normal human continually uses low-dose TCs, which increases the resistance of pathogens to antibiotics and leads to a serious threat to health. Hence, the determination of residual TCs in animal products is extremely important.

Nowadays, many traditional analytical techniques have successfully been employed to determine TCs, for example, flow injection analysis (FIA) [2], enzyme linked immunosorbent

✉ Fei Qu
  qufei3323@163.com

✉ Jinmao You
  jmyou6304@163.com

1 The Key Laboratory of Life-Organic Analysis, Qufu Normal University, Qufu, Shandong 273165, China

2 Key Laboratory of Pharmaceutical Intermediates and Analysis of Natural Medicine, Qufu Normal University, Qufu, Shandong 273165, China

3 Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining 810001, China

assay (ELISA) [5], high performance liquid chromatography (HPLC) [6], capillary electrophoresis (CE) [9], and electrochemical aptasensor [10]. These ways usually require expensive instruments, tedious sample extraction procedures and complicated preparation of sensors, although they are sensitive. However, nanotechnology brings useful perspective for TCs detection, which is simpler, rapider, more efficient, and much less costly, and these strategies are classified into two categories. (1) Direct detection is easily established. For instance, TET combines with the organic functional groups on the surface of carbon dots (CDs), and this leads to the quenching of the fluorescence of CDs [11]; on the basis of the above mechanism, OTC, CTC and DOX are measured by CDs [12, 13]. Besides, TCs induce the aggregation of gold nanoparticles with obvious color change. The change in the absorbance at 526 nm is directly correlated to the concentration of TCs [14]. (2) However, indirect detection is usually developed by virtue of the interaction between TCs and metal ions. For example, the chelation of OTC with Fe^{3+} suppresses fluorescence quenching of CDs by Fe^{3+} [15]; the combination of TET and Eu^{3+} enhances the fluorescence of metallic nanomaterials [4, 16]. In general, the indirect determination exhibits more sensitive but narrower linear range of TCs than direct detection.

In recent years, CDs attract intensive attention because of their unique optical properties, excellent water solubility, nontoxicity and good biocompatibility [17, 18]. On the basis of these superior properties, CDs provide a new platform for various optical applications. For instance, CDs are demonstrated to enable the detection of metal ions [19–21]; Garcia et al. employ CDs to detect 4-nitrophenol, which quenches the fluorescence of CDs [22]; CDs synthesized by citric acid and urea is used for fluorescence quantification of hypochlorite and peroxynitrite [23]. Furthermore, biothiols, such as cystein, glutathione and homocysteine, recover the fluorescence of the CDs, which is quenched by Cu^{2+} [24]. Compared with the traditional nanomaterials with a single emission, recent advances in the development of dual emission fluorescent nanomaterials provide promising candidates to construct novel optical sensors with improved properties. In Rao's work, they develop a selective and sensitive fluorescence sensor for ratiometric detection of Cu^{2+} by simply mixing blue-emission silica-coated CDs and red-emission thioglycolic acid modified CdTe quantum dots [25].

Herein, utilizing dually emitting carbon dots (de-CDs), which contain two emitters due to the different modified ligands on their surfaces, such as ascorbic acid (AA) capped on the blue emitters (BE) and alcohol molecules capped on the yellow emitters (YE) [26], a strategy integrating direct and indirect measurement of TCs is developed. TCs, including TET, OTC, CTC, and DOX directly quench the two emissions of de-CDs. This direct determination is simple, rapid and shows wide linear range. However, this method cannot distinguish these TCs and the sensitivity is not satisfactory. In the

subsequent experiment, on the basis of the fluorescence enhancement of YE produced by Al^{3+} [27], TET specifically quenches the fluorescence of the YE-Al^{3+} system due to the chelation of TET and Al^{3+}. This reveals a highly selectivity for TET over other antibiotics, especially OTC, CTC, DOX. The indirect detection also exhibits good sensitivity of TET with linear range from 1 nM to 30 μM (the limit of detection 0.52 nM), which is better than the reported literature [9–14, 16]. Benefiting from the superior selectivity and sensitivity, this indirect approach is successfully applied for the detection of TET in milk, fish and pork meat samples with good recoveries.

Experimental

Materials

Ascorbic acid (AA), ethylene glycol, $Al_2(SO_4)_3$, NaOH, H_2SO_4, methanol, ethylene diamine tetraacetic acid (EDTA), acetonitrile, tetracycline (TET), oxytetracycline (OTC), chlortetracycline (CTC), Na-penicillin G (Na-pen-G), erythromycin (ERY) and chloramphenicol (CHL) are purchased from Aladdin (Shanghai, China, http://www.aladdin-e.com). Doxycycline (DOX) and streptomycin sulfate (STR) are obtained from J&K Chemical Reagents Ltd. (Shanghai, China, http://www.jkchemical.com). All chemicals are analytical grade and use without further purification. Unless otherwise stated, ultrapure water is used throughout all of the experiments.

Instruments

The fluorescence spectra are recorded by a Hitachi F-7000 fluorescence spectrophotometer. The ultraviolet-visible (UV-vis) absorption spectra are performed on a Cary 300 Bio UV-vis spectrophotometer.

Preparation of de-CDs

The de-CDs are synthesized through one-pot hydrothermal method reported by our team recently [26]. In brief, 0.8 g AA is dissolved in ethylene glycol-water binary reaction media. All the volume of the mixture is 20 mL and the volume fraction of ethylene glycol is 50 %. Subsequently, under vigorous stirring to form a homogeneous solution, the mixture is heated to 160 °C in a constant temperature drying oven for 70 min. After cooling to room temperature, the mixture is centrifuged at 12,000 rpm (10,000×g) for 10 min. Finally, a clear yellow aqueous dispersion containing de-CDs is obtained, and the de-CDs are stored at ambient environment.

Fluorescence detection of TCs

(1) Direct detection Briefly, 0.0022 g TET is dissolved in H_2SO_4 (1.0 mL, 1.0 mol·L^{-1}), and the solution is diluted 10-fold in ultrapure water; the de-CDs solution is diluted 100 times before detection (10 μL·mL^{-1}). Then 60 μL of diluted TET, 30 μL (10 μL·mL^{-1}) de-CDs and ultrapure water are mixed under vigorous stirring. After 10 min of reaction time at ambient temperature, the fluorescence spectra are recorded at the excitation wavelength of 315 nm when BE is used as a probe. Additionally, 60 μL of diluted TET, 5 μL de-CDs without diluted and ultrapure water are mixed together. About 10 min later, the mixture is subjected to fluorescence measurement with excitation at 365 nm for YE as a probe. Additionally, the detection of OTC, CTC and DOX are followed by the same process as that of TET. Finally, the fluorescence intensities of de-CDs in the absence of TCs (F_0) and in the presence of TCs (F) are recorded, respectively. The quenching efficiency is calculated according to the relative fluorescence intensity (F_0-F)/F_0.

(2) Indirect detection Typically, different concentrations of TET (0–50 μM), Al^{3+} solution (100 μL, 200 μM) and ultrapure water are mixed together with vigorous stirring. One hour later, 5 μL de-CDs without diluted are added to the mixtures, and then these solutions are equilibrated at room temperature for 10 min. The fluorescence spectra of the mixtures are recorded with the excitation wavelength at 365 nm for YE as a probe. Moreover, the efficiency of fluorescence quenching is expressed as (F_1–F_2)/F_1, in which F_1 and F_2 represent the fluorescence intensities of the YE-Al^{3+} assay in the absence and presence of TET, respectively.

Treatment of real samples

Milk samples are obtained from the local supermarket. Briefly, 2.0 mL milk samples and 4.0 mL ultrapure water are transferred into a centrifuge tube with vigorous stirring. Then the sample is heated to 80 °C in a water bath for 20 min [15]. After cooling to room temperature, the mixture is centrifuged at a speed of 20,000 rpm (16,667×g) for 10 min. Subsequently, 0.2 mL acetonitrile is added into a moderate volume of supernatants with vigorous mixing [7]. After filtering through a 0.22 μm membrane to remove proteins [15], the mixture is adjusted to pH 6 with NaOH. Finally, three different concentrations (0.05, 1, and 20 μM) of standard TET solutions, 20 μM of Al^{3+} and 5 μL of undiluted de-CDs are added into the milk samples with pretreatment to further analyze, respectively.

Fish and pork meat samples are purchased from the local supermarket. At first, a weighed fish (1 g) is cut into very small pieces. After adding 5 mL methanol:H_2O (70:30, v/v) and 0.2 mL EDTA (0.1 M), the mixture is homogenized for 3 min; subsequently, the mixture is placed in an ultrasonic bath for about 1 h; then it is centrifuged at 3500 rpm (4200×g) for 5 min [28]. Additionally, 1 mL acetonitrile and 3 mL H_2O are added into a moderate volume of supernatants with vigorous mixing to remove proteins; after centrifugation again, the deproteinized fish sample is kept in a water bath for about 1 h to evaporate acetonitrile [7]. Finally, a series of pretreatment fish samples are spiked with the standard TET solutions (0.03, 3, and 30 μM) to further analyze. The preparation of pork meat samples is identical to that of fish.

# Results and discussion

Direct detection of TCs

Previously, our group successfully synthesized and characterized the de-CDs in detail [26]. The diameters of them range from 1 to 3 nm with an average diameter of about 1.81 nm; oxygen-containing functional groups such as hydroxyl and carbonyl groups on the surfaces of the two emitters ensure the good water solubility. As displayed in Fig. S1, the de-CDs contain two emitters: The BE exhibits the maximum emission at 386 nm with the excitation wavelength of 315 nm, and YE shows a maximum in 530 nm when excitation is 365 nm; correspondingly, the UV − vis absorption spectra indicate that the de-CDs show a strong peak at 256 nm and a weak peak at 370 nm [26].

As shown in Fig. 1, the two emitters of de-CDs are directly quenched by TET, OTC, CTC and DOX in 30 μM concentrations within 10 min. The photographs exhibit obvious fluorescence quenching of de-CDs with the addition of these TCs under ultraviolet lamp (UV lamp) at 365 nm (Fig. 1c). Moreover, this direct strategy displays high selectivity to TCs comparing with other antibiotics, including Na-penicillin G (Na-pen-G), erythromycin (ERY), chloramphenicol (CHL) and streptomycin sulfate (STR) (Fig. S2). In addition, the detailed linear ranges and the limit of detections (LODs) of TCs in direct measurement are given in Fig. S3 and Table S1. In general, the direct strategy possesses several advantages, such as simplicity, fast response, and low-cost; however, TET, OTC, CTC and DOX cannot be distinguished, and the sensitivity is not satisfactory; in the next step, an indirect strategy is developed based on YE-Al^{3+} system to detect TET with high selectivity and sensitivity.

Indirect detection of TET based on YE-Al^{3+} system

In our previous report, influences of metal ions on the fluorescence intensity of YE were discussed [27]. Bi^{3+} leads to the fluorescence quenching of YE, and the effect of Al^{3+} on YE is recognized by the remarkable enhancement of fluorescence with an obvious blue shift in emission (Fig. 2); however, other

Fig. 1 Fluorescence spectra of de-CDs in the absence and presence of TCs (**a**, BE; **b**, YE) and the corresponding photographs under UV lamp at 365 nm (**c**)
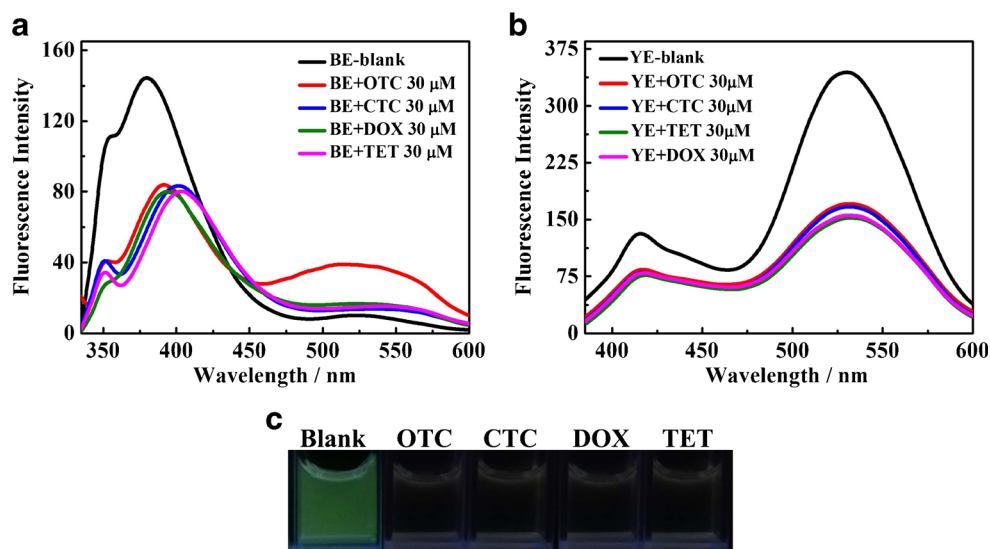
metal ions, such as Mn^{2+}, Fe^{2+}, Ce^{3+}, Ag^+, Ni^{2+}, Cu^{2+}, Mg^{2+}, Cr^{3+}, K^+, Na^+, Pb^{2+}, Sr^{2+}, Zn^{2+}, Co^{2+}, Ba^{2+}, Hg^{2+}, Fe^{3+} and Eu^{3+}, have no response to YE (Fig. S4). Additionally, there is no reaction between Bi^{3+} and TET, so a unique reaction between YE and Al^{3+} is used to detect TET as depicted in Fig. 2. When adding TET with low concentration (0.1 μM), the fluorescence quenches effectively, and the emission shifts to long-wavelength again; however, in absence of Al^{3+}, upon addition of same concentration of TET (0.1 μM), the fluorescence of the mixture of YE and TET is identical to that of YE. The photographs under UV light (Fig. 2b) coincide with the fluorescence spectra: The probe exhibits a bright yellow fluorescence, which is identical to that of YE-TET mixture in

absence of Al^{3+}; the fluorescence color changes to an obvious green after adding Al^{3+}; with addition of TET, the fluorescence of YE-Al^{3+} mixture decreases clearly. The distinct variation of fluorescence color suggests the feasibility and convenience of the indirect strategy. However, under the same concentration (0.1 μM), the OTC, DOX and CTC hardly quench the fluorescence of the YE-Al^{3+} system (Fig. S5).

Optimizing experimental conditions of indirect detection

In order to obtain a highly sensitive response of TET, the reaction conditions are discussed, such as the concentrations of Al^{3+}, probe concentrations, pH values, reaction times, reaction temperatures and addition orders (Fig. S6-S9). In Fig. S6, the fluorescence of YE enhances gradually with the increase of Al^{3+}; however, excessive Al^{3+} is disadvantageous to the sensitive detection of TET, so 20 μM Al^{3+} is chosen for the total experiments (Fig. S7a). Besides, 5 μL·mL^{-1} of probe is regarded as the optimal concentration and the proper reaction temperature is 25 °C (Fig. S7b and 7c). For YE, the stable fluorescence is observed in the pH range from 3 to 9 [26], thus the influence of pH is studied in this stable pH range and the optimal pH value is 6 (Fig. S7d). Furthermore, the reaction procedure between Al^{3+} and TET plays an important role in the fluorescence response (Fig. S8): Al^{3+} and TET should be pre-incubated, and then the probe is added. The optimal reaction time between Al^{3+} and TET is 1 h (Fig. S9).

Sensitivity of indirect detection

In Fig. 3, the fluorescence of YE produced by Al^{3+} decreases gradually with increasing concentrations of TET. This indicates that TET coordinates to Al^{3+}, which in turn causes fluorescence to be quenched and emission to be red-shifted.
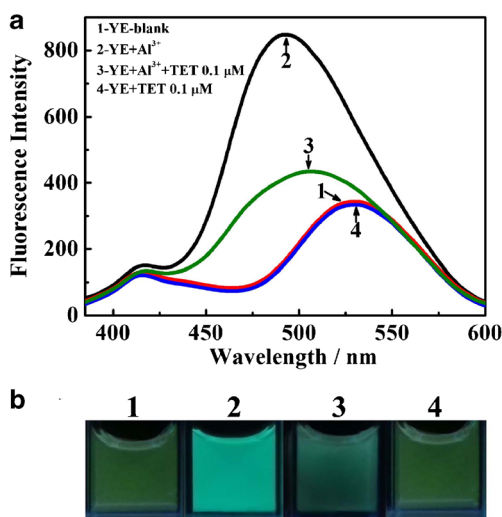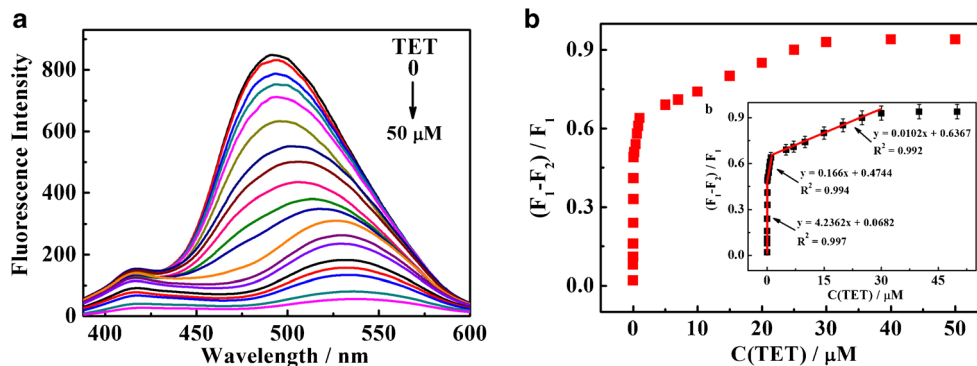

Fig. 2 Fluorescence spectra of free YE (1) and YE in the presence of 20 μM Al^{3+} (2), both of 20 μM Al^{3+} and 0.1 μM TET (3), and 0.1 μM TET (4), respectively (**a**), and the corresponding fluorescence of these solutions under UV lamp at 365 nm (**b**)

Fig. 3 Fluorescence spectra (**a**) of YE-Al^{3+} system in the presence of different concentrations of TET and the corresponding linear range of TET (**b**)

Therefore, a good linear relationship is established in the concentrations of TET from 1 nM to 30 μM with LOD 0.52 nM. For reproducibility study, the standard relative deviation ($n = 3$) of experimental data is controlled within 0.17 %. Especially, if the concentration of TET is 0.1 μM, the quenching efficiency of the direct detection is <0.07 %, while it can reach as much as 48 % in the indirect strategy. Hence, the sensitivity of indirect determination is much better than that of direct measurement. Besides, by comparison with the reported literature for the quantification of TET (Table 1), this method is demonstrated to be superior to other assays.

Selectivity of indirect detection

To estimate the selectivity of YE-Al^{3+} system for TET, the influence of other antibiotics including CTC, OTC, DOX, STR, ERY, CHL and Na-pen-G is studied in Fig. 4. The YE-Al^{3+} system exhibits a remarkable quenching of fluorescence toward TET; however, there is no apparent response in fluorescence intensity when given amounts of OTC, DOX, CTC, ERY, CHL, Na-pen-G and STR are present in the solution. Hence, this indirect strategy can distinguish TET from other TCs and exhibits excellent selectivity, even though these TCs possess similar structures (Fig. S10). This suggests that the YE-Al^{3+} system can serve as a unique fluorescent probe for determination of TET.

Indirect detection of TET in real samples

In order to investigate the applicability of indirect assay, the fluorescent probe of YE-Al^{3+} system is used to detect TET in milk, fish and pork meat samples. As exhibited in Table S2, the recoveries of TET are ranged from 91.7 to 102 % in these real samples. Hence, this method is sensitive, practicable and reliable, and can be used for the determination of TET in milk, fish and pork meat samples.

Mechanism of assay

(1) Direct detection In Fig. S11, the absorption spectra of TCs exhibit wide bands in the range from 200 to 400 nm, and they overlap with the excitation of BE and YE, so inner filter effect (IFE), which occurs effectively only if the absorption band of the absorber overlap with the excitation band of the fluorophore [29, 30], is the main mechanism for the fluorescence quenching of TCs in the direct detection. Besides, the absorption spectra of TCs also display weak overlap with the emission spectra of BE, thus fluorescence resonance energy transfer (FRET) is considered to be a partial quenching reason of BE [31], and in this procedure, BE acts as an energy donor, while TCs are acceptors. Therefore, under high concentrations of TCs, the fluorescence quenching mechanism of direct detection for BE is a combined effect of IFE and FRET, and for YE is the consequence of IFE.

Table 1 Comparison of different methods for the determination of TET

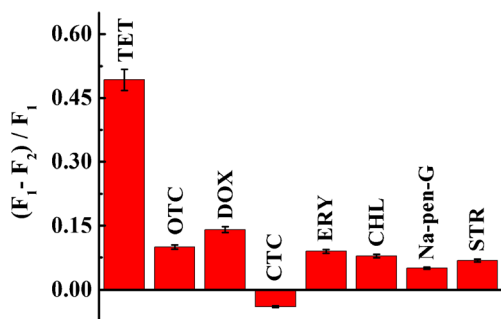| Methods	Linear range (μM)	LOD (μM)	References
CE	0.011–0.45	0.0038	9
Electrochemical aptasensor	0.01–50	0.005	10
Fluorescence	0.25–150	0.05	11
Fluorescence	0.01–100	0.0033	12
Fluorescence	0.06–8	0.0075	13
Colorimetry	0.97–29	0.38	14
Fluorescence	0.01–5	0.004	16
Fluorescence (indirect detection)	0.001–30	0.00052	This work

Fig. 4 Selectivity of YE-Al^{3+} system for the indirect detection of TET over other antibiotics. The concentration of TET is 0.1 μM, and the concentrations of other antibiotics (from left to right) are 1, 1, 1, 10, 20, 20 and 30 μM, respectively

As displayed in Fig. S12, for STR, a weak UV-vis absorption peak at 225 nm does not overlap with the excitation of BE and YE, while Na-pen-G and ERY do not possess UV absorbance in the range from 200 to 600 nm, thus these antibiotics cannot interfere with the detection of TCs in direct strategy. However, CHL shows a little spectral overlap with the excitation spectrum of BE and has no effect on the excitation and emission of YE, so CHL does not quench the BE effectively.

(2) Indirect detection TET possesses dimethylamino (C-4) and phenolic diketone (C-11; C-12) groups (Fig. S10a) [32]. Under acid condition, one molecule of water is removed from the C5a-C6 positions of TET [33]; TET undergoes reversible epimerization by changing the position of the C4-dimethylamino group to form 4-epitetracycline (4-epi-TET) at pH values between 2 and 6 [34]. On the basis of the changed structure, the C11-carbonyl and C12-hydroxyl of 4-epi-TET are coordination sites which are favorable for forming strong complexes with Al^{3+} in solution [35]. Herein, the optimal pH value is 6.0, so the chelation of Al^{3+} and 4-epi-TET inhibits the interaction between Al^{3+} and YE, producing the fluorescence quenching of YE-Al^{3+} system. This reaction procedure is depicted in Scheme 1. Moreover, with the increasing concentration of TET, it combines with all of Al^{3+} in solution, so
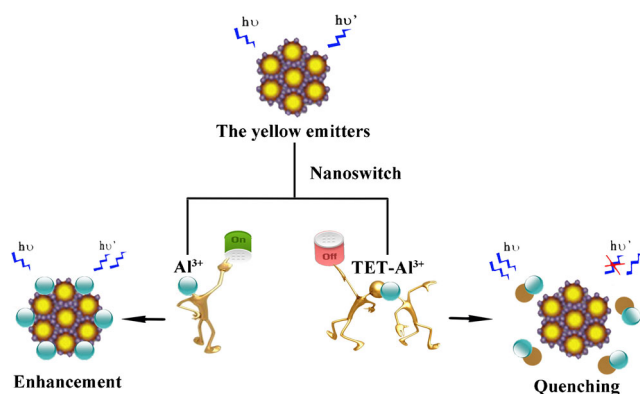
YE is released and the emission of YE shifts to long wavelength again; further increasing the concentration of TET, the direct interaction between TET and YE acts. Furthermore, it is doubt that other TCs are not able to quench the fluorescence of YE-Al^{3+} system even if they have similar structures with TET (Fig. S10). The reason may be that OTC and DOX have lower tendency to epimerize than TET at pH 6; their C5-hydroxyl group forms hydrogen-bonding with the C4-dimethylamino group [34], thus it is not conducive to the formation of OTC-Al^{3+} and DOX-Al^{3+} complexes. Moreover, CTC may also bind Al^{3+}; however, under higher concentration of CTC (3 μM), the complex (CTC-Al^{3+}) emits fluorescence at 490 nm as shown in Fig. S13, and this emission coincides with that of YE-Al^{3+} system, so the fluorescence of YE-Al^{3+} system cannot quench with addition of CTC; when the concentration of CTC is less than 1 μM, CTC may not interfere with the detection TET. Therefore, the selectivity of the indirect detection depends on the combination between TET and Al^{3+}, resulting in the fluorescence quenching of YE-Al^{3+} system.

Conclusions

Herein, we successfully establish a strategy combining direct and indirect detection of TCs based on de-CDs. High concentrations of TCs reduce the two emissions of de-CDs by IFE and FRET, while this strategy may not show special selectivity towards a certain kind of TCs. On the basis of YE-Al^{3+} system, the indirect detection of TET exhibits an excellent selectivity over other antibiotics, especially OTC, CTC, DOX, and a good linear relationship of TET is obtained. By taking advantages of the superior sensitivity and selectivity, the indirect assay is successfully applied to detect TET in milk, fish and pork meat samples with good recoveries. Therefore, this YE-Al^{3+} assay can serve as a potential candidate for detection of TET in animal food products.

Scheme 1 Indirect detection of TET based on the YE-Al^{3+} system

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