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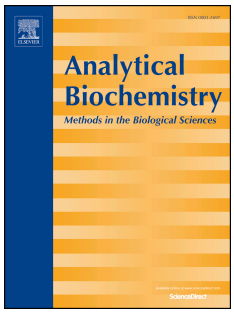

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Upconversion luminescence resonance energy transfer based aptasensor for the sensitive detection of oxytetracycline

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Short title: A FRET aptasensor for oxytetracycline detection

Subject category: Labeling Procedures

Abstract

In this work, a biosensor based on luminescence resonance energy transfer (LRET) from $NaYF_4$: Yb, Tm upconversion nanoparticles (UCNPs) to SYBR Green-I has been developed. The aptamers are covalently linked to UCNPs and hybridized with its complementary strands. The subsequent addition of SYBR Green allows SYBR Green-I to insert into the formed double-stranded deoxyribonucleic acid (dsDNA) duplex and brings the energy donor and acceptor into close proximity, leading to the fluorescence of UCNPs transferred to SYBR Green-I . When excited at 980 nm, the UCNPs emit luminescence at 477 nm, and this energy is transferred to SYBR Green-I, which emits luminescence at 530 nm. In the presence of oxytetracycline (OTC), the aptamers prefer to bind to its corresponding analyte and de-hybridize with the complementary DNA. This dehybridization leads to the liberation of SYBR Green-I, which distances SYBR Green-I from the UCNPs and recovers the UCNPs luminescence. Under optimal conditions, a linear calibration is obtained between the ratio of I_{530} nm to I_{477} nm (I_{530}/I_{477}) and the OTC concentration, which ranges from 0.1 ng/mL to 10 ng/mL with a limit of detection (LOD) of 0.054 ng/mL.

Keywords: Oxytetracycline, aptamer, luminescence resonance energy transfer, upconversion nanoparticles

1. **Introduction**

Oxytetracycline (OTC), a derivative of tetracyclines, is a broad-spectrum antibiotic that is widely used for the treatment or prevention of infections in bovine, pig, poultry, and fish productions [1]. It has become the most common growth promoter for livestock and aquaculture because of its low cost and high efficiency. However, the excessive use of tetracycline antibiotics may lead to the contamination of foodstuffs and the environment with the residues of these compounds. Indeed, antibiotic residues have been prohibited in foods because of their allergenic properties. Therefore, reliable analytical methods are required for monitoring OTC.

Traditionally, chromatographic methods, including high-performance liquid chromatography (HPLC) [2,3] and liquid chromatography-tandem mass spectrometry (LC-MS-MS) [4,5], have been used for the detection of tetracyclines in food products. These strategies are highly sensitive and specific, but require time-consuming pre-treatment steps and expensive instrumentation. Immunoassay methods using antibodies have also been applied because of their simplicity and highly sensitive and specific detection [6,7], but the preparation of the antibodies via animal immunization is time-consuming (several months), and the antibodies may become susceptible to stability or modification issues.

To overcome the limitations described above, new synthetic receptors known as aptamers have been explored as highly promising alternatives. Aptamers are single-stranded nucleic acid molecules that are selected via a process termed SELEX (Systematic Evolution of Ligands by Exponential Enrichment) [8,9]. These selected aptamers can bind a wide range of targets and the affinities of aptamers for their targets are comparable to, or even higher than those of most monoclonal antibodies. Moreover,

aptamers can be chemically labeled facilitating the incorporation of these novel receptors in biosensing devices for a broad range of applications [10]. Biosensors based on aptamers for OTC detection have been developed, such as colorimetric aptasensors [11,12], electrochemical aptasensors [13], and fluorescence aptasensors [14].

Förster resonance energy transfer (FRET), a direct and sensitive technology, has been widely used for bioassays, biosensing, bioimaging, and photodynamic therapy [15-17]. The use of appropriate donor-acceptor pairs is extremely critical for improving the efficiency of FRET and the resulting analytical performance. It is known that lanthanide-based upconversion nanoparticles (UCNPs) can convert low-energy near-infrared (NIR) photons into visible emission by sequentially absorbing multiple photons and undergoing energy transfer; this process is known as luminescence resonance energy transfer (LRET) [18]. Compared to down-conversion fluorophores, UCNPs shows excellent optical features, such as low phototoxicity, low autofluorescence background, and large Stokes shifts. Additionally, UCNPs also have high chemical stability and excellent photostability. Recently, UCNPs based LRET biosensors have demonstrated promising applications in detecting biomolecules, such as DNA and proteins [19,20]. However, there are limited reports of UCNPs-based LRET biosensors for OTC detection.

SYBR Green, a fluorescent dye, exhibits very weak fluorescence when it is unbound. However, this dye molecule becomes highly fluorescent upon binding to dsDNA but exhibits no significant fluorescence when binding to single-stranded DNA. This property makes SYBR Green very useful in label-free biosensors.

In this work, a $NaYF_4$: Yb, Tm UCNPs-based LRET biosensor has been developed, with $NaYF_4$: Yb, Tm UCNPs and SYBR Green-I as the energy donor-acceptor pairs.

Aptamers targeted to OTC were first conjugated to UCNPs. The LRET process was initiated by the addition of SYBR Green-I and the hybridization between the aptamers and their complimentary strands. When excited at 980 nm, the UCNPs emit luminescence at 477 nm, and this energy is then transferred to SYBR Green-I, which emits luminescence at 530 nm. This emission can be used to quantify OTC.

2. Materials and method

2.1 Reagents and chemicals

The rare earth nitrates used in this work, including Y $(NO_3)_3 \cdot 6H_2O$, Yb $(NO_3)_3 \cdot 5H_2O$, and Tm $(NO_3)_3 \cdot 5H_2O$, were of 99.99% purity and were purchased from Aladdin Industrial Inc. (Shanghai, China). Oleic acid, cyclohexane, 25% ammonia, 25% glutaraldehyde ($OHC(CH_2)_3CHO$), tetraethyl orthosilicate (TEOS) and ethanol were of analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd.(Shanghai, China). IGEPAL CO-520, 98% 3-aminopropyltrimethoxysilane (APTES) was purchased from Alfa Assar (USA). The OTC aptamer [21] and its partially complementary strand were synthesized by Shanghai Sangon Biological Science & Technology Company (Shanghai, China). The sequence of the OTC aptamer was 5’-NH_2-GGAATTCGCTAGCACGTTGACGCTGGTGCCCGGTTGTGGTGCGA GTGTTGTGTGGATCCGAGCTCCACGTG-3’(aptamer), and the sequence of its partially complementary strand was 5'-NH_2-CAACGTGCTAGCGAA-3'(cDNA). OTC, tetracycline (TET), and doxycycline (DOX) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China).

2.2 Apparatus

The size and morphology of the nanoparticles were observed on a JEM-2100HR transmission electron microscope (TEM, JEOL Ltd., Japan), using an accelerating voltage of 200 kV. X-ray diffraction (XRD) measurements were performed using a D8-advance instrument (Bruker AXS Ltd., Germany) with graphite-mono-chromatized Cu-Kα radiation (λ=0.15406 nm). The luminescence spectra of UCNPs were measured on an F-7000 luminescence spectrophotometer (Hitachi Co., Japan) attached to an external 980 nm laser (Beijing Hi-Tech Optoelectronic Co., China) instead of the internal excitation source. FTIR spectra of the amino-modified NPs were measured with a Nicolet Nexus 470 Fourier transform infrared spectrophotometer (Thermo Electron Co., USA) using the KBr method. Ultraviolet-visible (UV-vis) absorption spectra were recorded using a Shimadzu UV-1800 UV-vis spectrophotometer (Shimadzu, Japan).

2.3 Synthesis and surface modification of rare earth-doped $NaYF_4$: Yb, Tm UCNPs

Oleic acid-capped UCNPs were prepared by a modified hydrothermal process. In a typical procedure for the synthesis of $NaYF_4$: Yb, Tm UCNPs, NaOH (1.2 g, 30 mmol), water (9 mL), ethanol (10 mL), and oleic acid (20 mL) were mixed under agitation to form a homogeneous solution. Next, 0.936 mL of 0.5 M $Y(NO_3)_3$, 0.6 mL of 0.2 M $Yb(NO_3)_3$, and 0.06 mL of 0.2 M $Tm(NO_3)_3$ were added to the mixture and stirred thoroughly. Subsequently, a 0.168 g NaF (4 mmol) solution was added dropwise to the previously prepared solution. After being vigorously stirred at room temperature for 15 min, the colloidal solution was transferred into a 50 mL Teflon-lined autoclave, sealed, and heated at 190 °C for 12 h. After the mixture cooled to room temperature, the products were precipitated by adding ethanol to the bottom of the vessel, centrifuged to obtain a powdered sample, and washed with ethanol and distilled water several times.

Surface modification of the $NaYF_4$: Yb, Tm UCNPs was achieved using a microemulsion method to cap silica onto the surface of the UCNPs [22]. Then, 500 µL of CO-520, 10 mL of cyclohexane, and a 2 mL volume containing 2 mg of $NaYF_4$: Yb, Tm UCNPs nanospheres were mixed and stirred for 10 min. Then, ammonia was added, and the solution was sonicated for 20 min until a transparent emulsion was formed. Subsequently, 100 µL of TEOS and 20 µL of APTES were added to the solution, and the solution was rotated for two days at a speed of 600 rpm. Silica/$NaYF_4$ nanospheres were precipitated by adding acetone, and the nanospheres were washed with ethanol/water.

2.4 Attachment of aptamer to the UCNPs

The procedure for the preparation of aptamer-conjugated UCNPs (aptamers-UCNPs) was adapted from the classical glutaraldehyde method. $NaYF_4$:Yb, Tm UCNPs (1 $mg \cdot mL^{-1}$) and 0.2 mL of 25% glutaraldehyde were shaken slowly on a shaking table at room temperature for 2 h. The $NaYF_4$: Yb, Tm UCNPs were separated by centrifugation and washed with PBS three times. The resulting glutaraldehyde-activated UCNPs were covalently linked with 5 µL of the 5'-NH_2 modified aptamer (100 µM). The reaction was carried out under gentle mixing for 12 h. Finally, the UCNPs tagged with aptamer were harvested by centrifugation.

2.5 LRET and the OTC detection

A total of 200 µL of aptamer-UCNPs was hybridized in PBS buffer with 2.5 µL of cDNA (100 µM) at 37 °C for 1 h to achieve aptamer-cDNA hybridization. Subsequently, 35 µL of 20×SYBR Green-I was added to the aptamer-cDNA duplex. The mixture was

shaken slowly on a shaking table at room temperature for 10 min in the dark. Then, various concentrations of OTC were added to the mixture and further incubated at 37 °C for 45 min. The luminescence intensity of the liquor was measured with a 980 nm excitation laser.

2.6 Method comparison by ELISA in food samples and recovery experiments

The accuracy of OTC detection in food samples was evaluated by determining the recovery of OTC (Recovery ratio=(detected concentration-background content)/added concentration). Milk samples were pretreated by centrifugation separation (10 °C, 7000 rpm) for 10 min, and the upper layer of cream was removed. Subsequently, the milk was diluted with PBS buffer (1:10 v/v). A series of known quantities of OTC were added into the milk samples for the recovery experiments. The commercially available ELISA method was applied to detect OTC in the same milk samples.

3. Results and Discussion

3.1 Detection principle

We presented a new aptasensor for OTC that was based on LRET between UCNPs as donors and SYBR Green-I as an acceptor. The luminescence bioassay platform for the detection of OTC based on LRET is illustrated in Fig. 1. Briefly, OTC aptamers were first conjugated to UCNPs to form aptamer-UCNPs complexes. Then, the aptamers-UCNPs were hybridized with cDNA to form a duplex structure. The subsequently added SYBR Green-I inserted into the dsDNA, which caused the energy donor and acceptor to come into close proximity, which led to the transfer of the UCNPs' luminescence to SYBR Green-I. When excited at 980 nm, the intensity of the

UCNPs emission peak at 477 nm was quenched, and a new emission peak at 530 nm which corresponded to SYBR Green-I, appeared. This occurred because there is a good overlap between the luminescence emission of UCNPs and the excitation of SYBR Green-I. In the presence of OTC, the aptamers preferred to bind to its corresponding analyte and de-hybridize with the complementary DNA, thereby liberating some SYBR Green-I. Finally, the luminescence intensity of UCNPs at 477 nm was recovered because the UCNPs and SYBR Green-I were widely separated. Thus, the concentration of OTC could be quantified according to the ratio between SYBR Green-I emission and that of UCNPs (I_{530}/I_{477}). Moreover, unlike traditional fluorescence quenchers, such as BHQ-1 and BHQ-2, which are used as DNA labels, SYBR Green is a label-free indicator and makes the detection very simple and convenient.

3.2 Preparation and characterization of UCNPs

The UCNPs were synthesized via a solvothermal method using nitrates. Fig. 2A shows representative TEM images of the oleic acid-capped UCNPs. As observed, the $NaYF_4$:Yb, Tm UCNPs are well-dispersed and uniform in size. The microemulsion method was used to coat the UCNPs with silica for TEM characterization (Fig. 2B). After silica coating, the nanocrystals were dispersible in water and demonstrated good chemical and photochemical stabilities. Moreover, no significant change in the luminescence intensity of the UCNPs was caused by the thin silica shell on the surface (Fig. 2C). Fig. 2D presents the XRD patterns of the $NaYF_4$: Yb, Tm UCNPs, revealing that the prepared UCNPs include both hexagonal-phase (JCPDS no. 16-0334) and cubic-phase nanocrystals (JCPDS no. 77-2042). The functional groups on the surfaces of the amino-modified $NaYF_4$: Yb, Tm UCNPs were identified by FT-IR spectra, as

shown in Fig. 2E. The wide absorption peak at 3142 cm^{-1} (curve a) is the stretching vibration of the hydroxyl group. Two peaks at 1560 and 1458 cm^{-1} correspond to the asymmetric and symmetric stretching vibrations of the carboxylic group (COO–). In addition, bands at 2921 and 2853 cm^{-1} are assigned to the asymmetric and symmetric stretching vibrations of the methylene group, respectively, caused by the long chain alkyl of the oleic acid molecules. These findings verified that UCNPs were coated with oleic acid molecules prior to midification. In contrast, the peaks mentioned above disappeared together (curve b), and the characteristic peaks corresponding to amino groups appeared instead. The hydroxyl stretching vibration band of a silanol group (Si–OH) appears approximately 3042 cm^{-1}, and the band at 1068 cm^{-1} is attributed to the stretching vibration of the Si–O bond. The peak at 1625 cm^{-1} is the stretching and bending vibration bands of the amino group, indicating that the silica-coated UCNPs were successfully functionalized with amino groups.

3.3 Characterization of aptamer-conjugated UCNPs

We applied UV-vis spectrophotometry to validate the successful coupling of amino-modified OTC aptamer to amino-modified nanoparticles. As shown in Fig. 3, no strong absorbance peak was detected for $NaYF_4$: Yb, Tm UCNPs (curve a). After conjugation to the aptamer, a new absorption peak at approximately 260 nm was observed (curve b). These results demonstrate that aptamer-$NaYF_4$: Yb, Tm UCNPs complexes were successfully formed.

3.4 Optimization of the experimental conditions

It should be noticed that the dosage of SYBR Green-I may strongly affect the

detection result. Thus, it is necessary to optimize the dosage of SYBR Green-I. Aptamer-UCNPs were hybridized with cDNA to achieve aptamer-cDNA hybridization. Subsequently, various volumes of 20× SYBR Green-I were added. The mixture was shaken slowly on a shaking table at room temperature. As shown in Fig. 4A, the maximum I_{530}/I_{477} ratio was achieved with the addition of 35 μL of SYBR Green-I.

In addition, the intercalating dyes require a period of incubation to completely generate the duplex structure. Therefore, we also optimized the reaction time for SYBR Green-I and aptamer-cDNA hybridization to obtain the best LRET effect. The time dependence of the assay was studied in the range of 0-20 min (Fig. 4B), and the I_{530}/I_{477} ratio was found to increase as the reaction time was increased until 10 min, after which the ratio plateaued and remained constant. Therefore, 10 min was chosen as the optimized intercalation time for the experiment.

3.5 Determination of OTC

In this experiment, the I_{530}/I_{477} ratio was maximized in the absence of OTC. When OTC was added to the system, the aptamer preferentially bound to OTC, causing the some dissociation of the aptamer from cDNA, and thereby liberating some SYBR Green-I. Thus, the intensity of the emission peak at 477 nm was recovered as a result of the disintegration of the LRET structure. Various intensities of luminescence were obtained in the presence of different concentrations of OTC, as shown in Fig. 5A. Fig. 5B presents the linear relationship between the I_{530}/I_{477} ratio and the concentration of OTC. The LOD of the aptasensor for OTC was as low as 0.054 ng/mL (calculated as 3s/S, where s is the standard deviation of the blank solution and S is the slope of the linear relationship). The precision, expressed in terms of the relative standard deviation

(RSD) of OTC detection was 2.5% (0.5 ng/mL, n=11).

3.6 Specificity evaluation and analytical application

Three structurally similar tetracycline derivatives, including OTC, DOX, and TET were used to verify the good selectivity of this method. The aptasensor were exposed to the derivatives at the same concentration (0.5 ng/mL). The results are shown in Fig. 6, OTC caused a dramatic change in the luminescence, as was expected, while the others failed to produce a similar change. The good specificity of this method was attributed to the inherent specificity of the aptamer for OTC.

The accuracy of OTC detection in food samples was evaluated by determining the recovery of OTC by adding a series of known quantities of OTC into the milk samples. As shown in Table 1, the recoveries were between 97.16% and 109.50%, and there was no significant difference ($P<0.0001$) between the results obtained by the aptasensor and the ELISA method, indicating that the developed LRET-based aptasensor can be applied for OTC detection in food samples.

4. Conclusion

A novel aptasensor was fabricated for the detection of OTC based on the luminescence resonance energy transfer (LRET) technique between upconversion nanoparticles (UCNPs) and SYBR Green dye. UCNPs can act as excellent emitters because of their low autofluorescence and high penetration depth in biosamples. SYBR Green can insert into the duplex structure formed by the hybridization of aptamer-UCNPs and cDNA, which caused the energy donor and acceptor to come into close proximity, leading to the transfer of luminescence of UCNPs to SYBR Green. In

addition, this system shows high specificity because of the properties of the aptamers. Therefore, the method is simple and convenient and could be applied for the determination of various targets in the future.

Acknowledgements

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Figure Captions

Fig. 1 Schematic illustration of the sensitive aptasensor for oxytetracyline based on upconversion luminescence resonance energy transfer

Fig. 2 TEM images of $NaYF_4$:Yb，Tm UCNPs before coating with silica (A) and after coating with silica (B), Luminescence spectra of $NaYF_4$: Yb, Tm nanospheres, before (black curve) and after were coated with silica (red curve) (C), XRD patterns of $NaYF_4$: Yb, Tm UCNPs (D), FT-IR spectra of oleic acid–capped $NaYF_4$ UCNPs (a) and silica coated $NaYF_4$ UCNPs (b) (E).

Fig. 3 UV-vis spectra of $NaYF_4$: Yb, Tm nanospheres (a), and aptamer conjugated $NaYF_4$: Yb, Tm nanospheres (b).

Fig. 4 I_{530}/I_{477} ratio obtained using various amounts of SYBR Green-I (A), and the effect of the incubation time of SYBR Green-I and aptamer-cDNA duplex on the I_{530}/I_{477} ratio.

Fig. 5 Typical recorded output for the detection of OTC (A), and standard curve of the relative luminescence intensity (I_{530}/I_{477}) versus OTC concentration (B).

Fig. 6 Differential luminescence responses of the aptasensor in terms of I_{530}/I_{477} to OTC, DOX, and TET at the same concentration (0.5 ng/mL).

Table 1 OTC detection using the developed method and the ELISA method.

Table 1 OTC detection using the developed method and the ELISA method.

Sample	Background Content (ng/mL)	Add Concentration (ng/mL)	Detected Concentration (ng/mL)		Recovery Ratio (%)
			ELISA	Developed method	
1	0	0.2	0.198	0.219	109.50
2	0	0.6	0.592	0.593	98.83
3	0	2	2.119	1.942	97.16
4	0	6	5.839	5.983	99.72
5	0	8	7.981	8.133	101.66

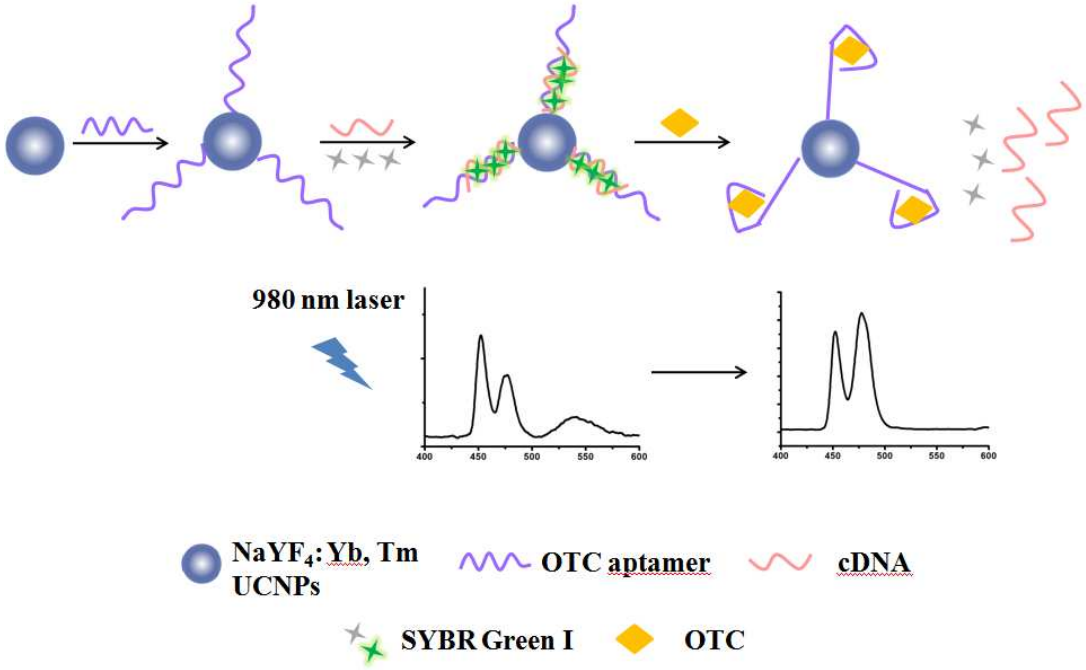
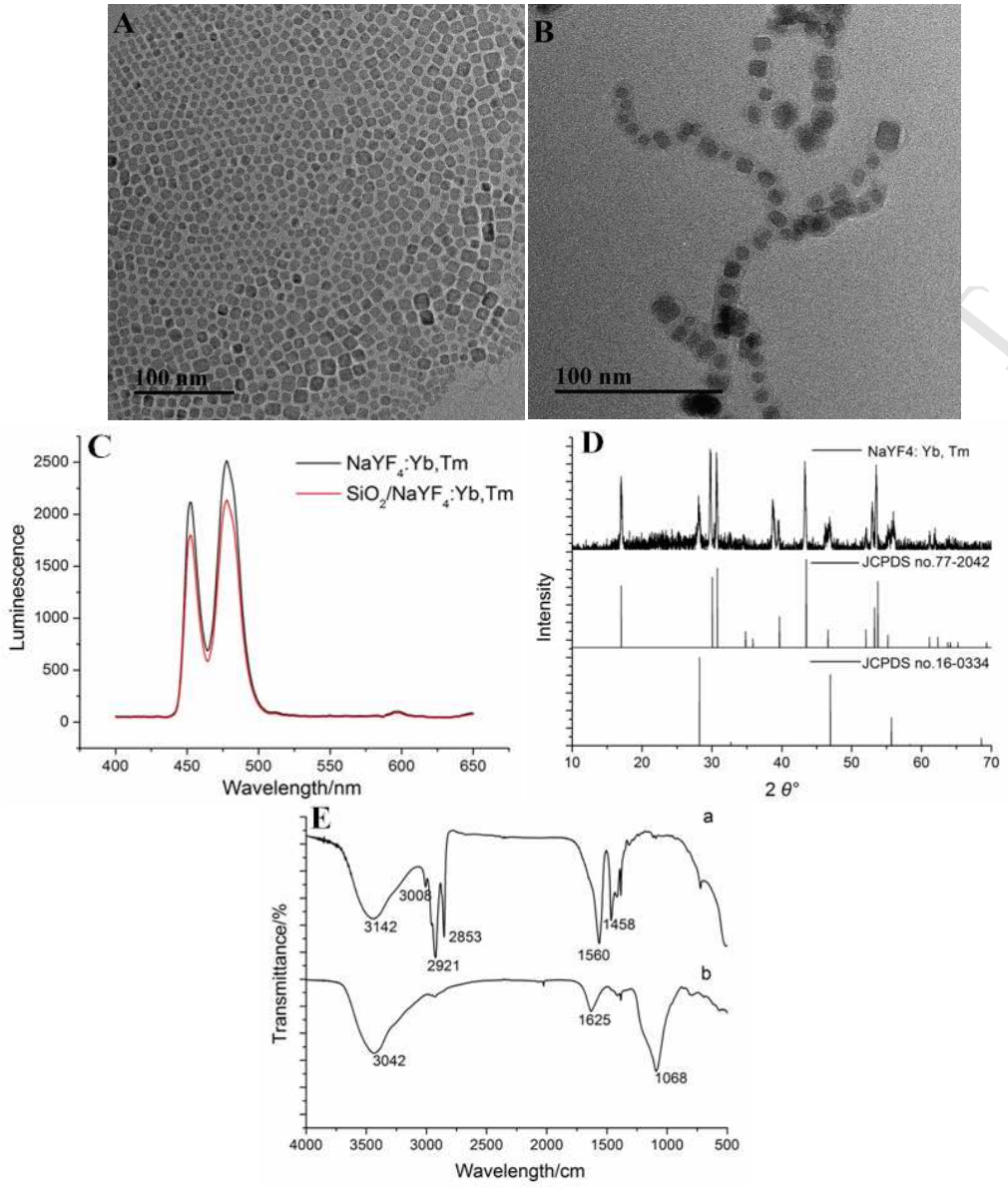


Fig. 1


A
100 nm
B
100 nm
C
NaYF4:Yb,Tm
SiO2/NaYF4:Yb,Tm
Luminescence
Wavelength/nm
D
NaYF4: Yb, Tm
JCPDS no.77-2042
JCPDS no.16-0334
Intensity
2 θ°
E
a
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b
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1068
Transmittance/%
Wavelength/cm

Fig. 2

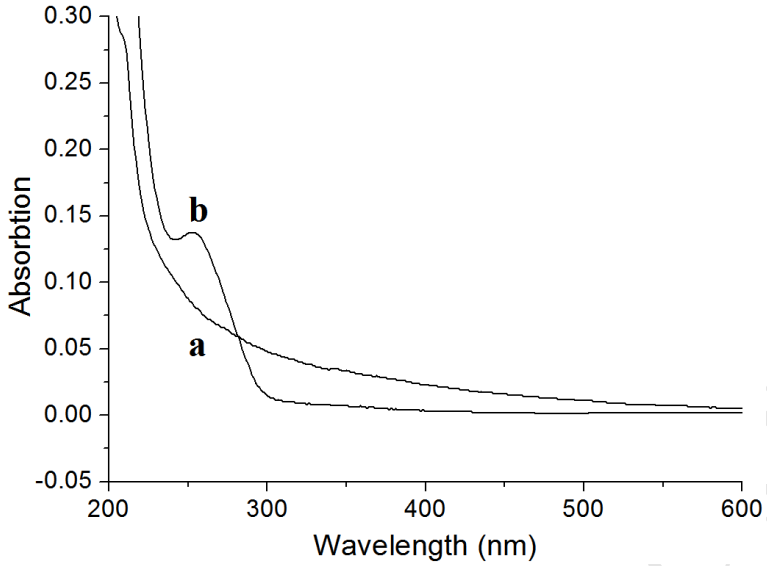

Fig. 3

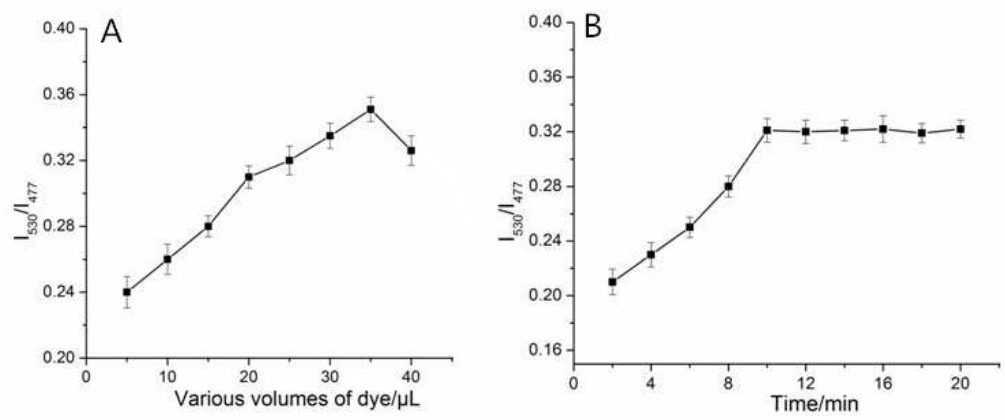

Fig. 4

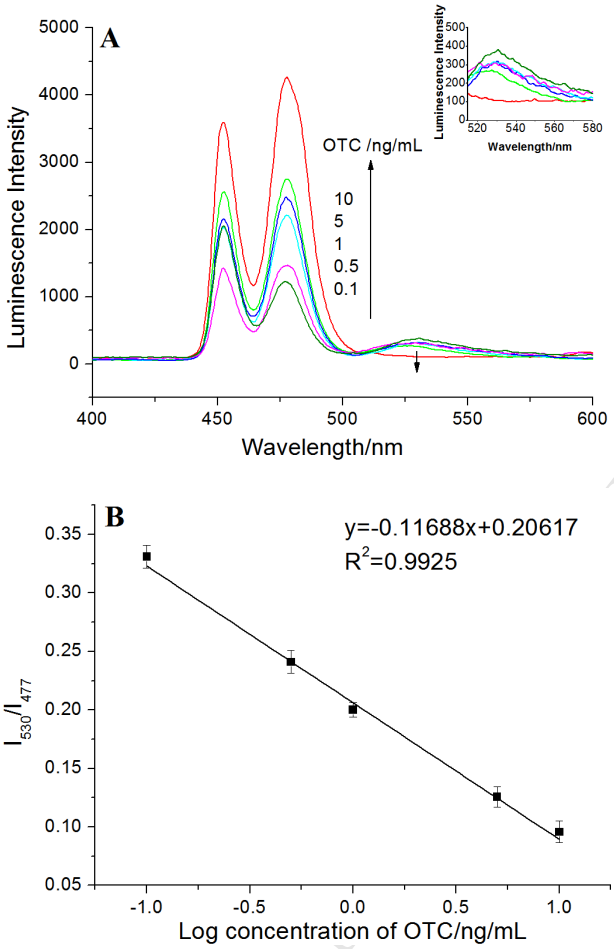

Fig. 5

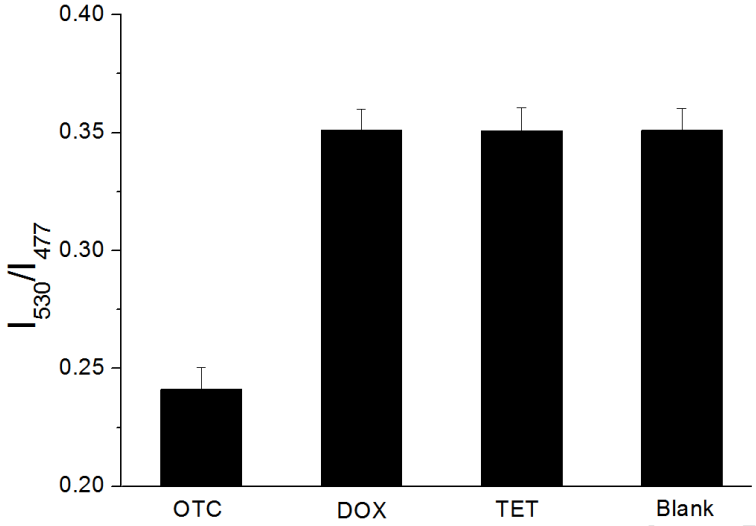

Fig. 6