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ORIGINAL PAPER

Homogeneous time-resolved FRET assay for the detection of *Salmonella typhimurium* using aptamer-modified $NaYF_4$: Ce/Tb nanoparticles and a fluorescent DNA label

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Abstract The authors describe an aptasensor based on time-resolved fluorescence resonance energy transfer (TR-FRET) for the identification of *Salmonella typhimurium* (*S. typhimurium*). Aptamer-functionalized nanoparticles (NPs) is used as the energy donor, and a complementary oligonucleotide (cDNA) labeled with carboxyfluorescein (FAM) acts as the acceptor. The detection scheme is based on the hybridization between aptamer and cDNA, upon which photonic energy is transferred from NPs to FAM unless aptamer interacts with *S. typhimurium*. Due to the highly specific recognition ability of aptamer and the strong fluorescence intensity of NPs, the method is highly sensitive and selective for *S. typhimurium*. Under the optimal conditions, at excitation wavelength of 273 nm, a delay time of 100 μs and a gating time of 1 ms, the integrated time-resolved fluorescence intensity ratio (FAM_{520}/Tb_{489}) is linear in the 100 to 10^6 cfu·mL^{-1} range, and the limit of detection is as low as 25 cfu·mL^{-1}. The assay was applied to the analysis of eggs and chicken meat for *S. typhimurium*, and the results were consistent with those of a plate-counting method.

Keywords Nanoparticles · Time-resolved fluorescence · Aptamer · Foodborne pathogen detection

Introduction

Salmonella typhimurium (S. typhimurium) is one of the leading causes of food-borne illness in human and animal hosts worldwide [1]. In United Stated almost million cases of salmonellosis with 19,000 hospitalizations and up to 300 deaths were caused by *S. typhimurium* each year [2]. In China, approximately 70 ~ 80% of food-borne bacteria outbreaks are thought to be caused by *Salmonella* and 90% of *Salmonella* was transmitted through the consumption of raw or uncooked vegetables, poultry, eggs, and fruits [3, 4]. Therefore, it is of great importance to develop new techniques with faster response time, better sensitivity and selectivity for *S. typhimurium* detection.

The existing methods for detecting *S. typhimurium* include conventional plate counting method, polymerase chain reaction (PCR) [5] and immunoassay methods [6]. Though the results of conventional method as well as PCR based method are accurate and reliable, the preparation work (such as pre-enrichment, selective enrichment, PCR amplification and/or cell culturing) is laborious, time consuming and unable to meet the rapid detection needs [7]. The immunoassay methods have become a powerful tool for biological research and clinical diagnostics, due to the convenient operation and the ability to test a large number of samples at the same time [8, 9]. However, these methods are heavily reliant on the quality of the antibodies, which are costly and susceptible to stability or modification issues.

To rival antibodies in these ways, aptamers with high affinity and selectivity are beginning to emerge and used for therapeutic

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and diagnostic applications. Aptamers are DNA or RNA molecules which can adopt specific three-dimensional conformations to combine with target analytes [10, 11]. Aptamers provide a variety of advantages over antibodies. For example, aptamers are stable, inexpensive, flexibly to chemically modify, and minimally immunogenic [12, 13]. In addition, aptamers can be modified with a variety of signal tags and designed as fluorescent [14], electrochemical [15], SERS [16] and colorimetric [17] sensors for a wide range of targets [18].

Fluorescence resonance energy transfer (FRET) is a typical and widely used homogeneous assay, due to its convenient experimental procedure [19, 20]. However, conventional FRET assays is severely compromised by autofluorescence interference, which limits practical application of the FRET technique. Time-resolved fluorescence resonance energy transfer (TR-FRET), which employing the long fluorescence emission lifetimes of lanthanide ion (Ln^{3+}) compounds, can effectively reduce the background interference from ubiquitous endogenous fluorescent components [21, 22] and thus offers a signal with remarkably high signal-to-noise ratio in fluorescence biodetection as compared to conventional FRET [23, 24]. Ln^{3+}-chelates as biolabels has been well developed and commercialized for decades [25]. However, most Ln^{3+}-chelates is susceptible to photobleaching, have low labeling ratio and high cost, which strongly restricts the sensitivity of the assay and its widespread applications. As an alternative to Ln^{3+} chelates, Ln^{3+}-doped inorganic nanoparticles possesses a series of advantages, such as high photochemical stability, excellent flexibility for bioconjugation, low cytotoxicity and low cost, thus can be used as a new fluorescence nano-bioprobes [26].

Herein, we introduce a TR-FRET method for rapid and sensitive detection of *S. typhimurium* using aptamer as a specific recognition element, time-resolved inorganic nanoparticles (NPs) as energy donor and fluorescent dye as acceptor. This method demonstrates the novelty use of long lifetime and strong fluorescence intensity of NPs and unique FRET between NPs and fluorescent dye for the quantitative analysis of pathogenic bacteria. In combination with the use of aptamer as recognition elements, this method shows high sensitivity, good selectivity and short detection time.

Materials and methods

Reagents

$Tb(NO_3)_3{\cdot}5H_2O$, $Ce(NO_3)_3{\cdot}6H_2O$ and $Y(NO_3)_3{\cdot}6H_2O$ were purchased from Aladdin Chemistry Co.Ltd. (China) (http://www.aladdin-e.com). 2-aminoethyl dihydrogen phosphate (AEP) was purchased from TCI (Shanghai) Development Co., Ltd. (http://www.tcichemicals.com). Ethylene glycol, ethanol, glutaraldehyde aqueous solution (25% in *V*/V), NaCl and the chemicals used to prepare the buffers and solutions were obtained from Sinopahrm chemical Reagent Co. Ltd. (China) (http://www.sinoreagent.com). Avidin was purchased from Sigma-aldrich Co. LLC (USA) (http://www.sigmaaldrich.com). The opaque 96-well microtitration microplates (300 mL/well) were purchased from Corning Inc. (USA) (http://www.corning.com).

The *S. typhimurium* aptamer was reported by Raghavendra Joshi et al. [27]. The DNA sequence of *S. Typhimuriu* aptamer is 5′-biotin-C6-TAT GGC GGC GTC ACC CGA CGG GGA CTT GAC ATT ATG ACA G-3′. Carboxyfluorescein (FAM) was used as fluorescent dye, and the complementary DNA (cDNA) sequence is 5′-GAC GCC GCC ATA-FAM-3′. The biotin labeled aptamer and FAM labeled cDNA were synthesized by the Shanghai Sangon Biological Science & Technology Company (Shanghai, China) (http://www.sangon.com).

Apparatus

The time-resolved fluorescence (TRFL) spectra was carried out on a multi-mode microplate reader with hybrid technology (Synergy H1, BioTek) upon excitation at 273 nm, where the delay time and gate time were set to be 100 μs and 1 ms, respectively. Power X-ray diffraction (XRD) pattern was collected using a D8-advance (Bruker AXS Ltd., Germany) with graphite-monochromatized Cu Kα radiation ($\lambda = 0.15406$ nm). TEM measurement was performed using a JEOL model 2100 HR instrument and operating at 200 kV accelerating voltage (TEM, JEOL Ltd., Japan). The infrared spectra of the bionanoparticles was collected using a Nicolet Nexus 470 Fourier transform infrared spectrophotometer (FTIR, Thermo Electron Co., U.S.A.) using the KBr method. Imaging of the aptamer-NPs (apt-NPs) connected with FAM-cDNA to construct NPs-FAM pair was performed with a ZEISS LSM 710 confocal microscope (Carl ZEISS, Germany). Ultraviolet-visible absorption spectra were recorded on a UV-1800 spectrophotometer (Shimadzu Co., Japan). The transient decay of the NPs was measured by employing an Edinburgh Instrument FLS920 spectrofluorometer equipped with both continuous (450 W) xenon and pulsed xenon lamp. The suspension of nanoparticles was prepared using an ultrasonic bath KJ-300 (Wuxi Kejie Electron Instruments Co. Ltd., China).

Synthesis of $NaYF_4$: Ce/Tb nanoparticles

$NaYF_4$: Ce/Tb was synthesized according to Tu's and our previously reported procedure with minor alteration [28, 29]. Briefly, AEP (1 mmol) and NaCl (1 mmol) were dissolved in 30 mL ethylene glycol, then $Y(NO_3)_3{\cdot}6H_2O$ (0.9 mmol), $Ce(NO_3)_3{\cdot}6H_2O$ (0.05 mmol) and $Tb(NO_3)_3{\cdot}6H_2O$ (0.05 mmol) were added under magnetic stirring to form a homogeneous solution. Subsequently, 10 mL ethylene glycol solution containing NH_4F (4 mmol) was added dropwise. The mixed solution was stirred for 30 min and was transferred to a

50 mL Teflon-lined autoclave and sealed and subsequently treated solvothermally at 180 °C for 4 h. After cooling to room temperature (RT), the products were collected by centrifugation, and washed with ethanol and water three times.

Preparation of signal probes

The procedure for preparation of avidin-modified NPs was adapted from the classical glutaraldehyde method according to our previous work [29]. In brief, 1 mg of $NaYF_4$: Ce/Tb NPs was first dissolved in 1 mL of phosphate buffered saline (PBS 137 mmol mL^{-1} NaCl, 2.7 mmol mL^{-1} KCl, 10 mmol mL^{-1} Na_2HPO_4, and 2 mmol mL^{-1} KH_2PO_4, pH 7.4) by ultrasonication for 15 min, and 250 μL of glutaradehyde solution was introduced to the mixture under gentle agitation for 3 h at 25 °C, the NPs was separated by centrifugation and were washed three times with PBS.

The 5′-biotin-labeled aptamer and avidin-functionalized NPs were bound via the high-affinity biotin-avidin system. Generally, the amount of the required 5′-biotin-labeled aptamer was introduced to the avidin-functionalized NPs solution and incubated for 6 h at 37 °C. The final products were washed with PBS three times, and redispersed in fresh PBS buffer.

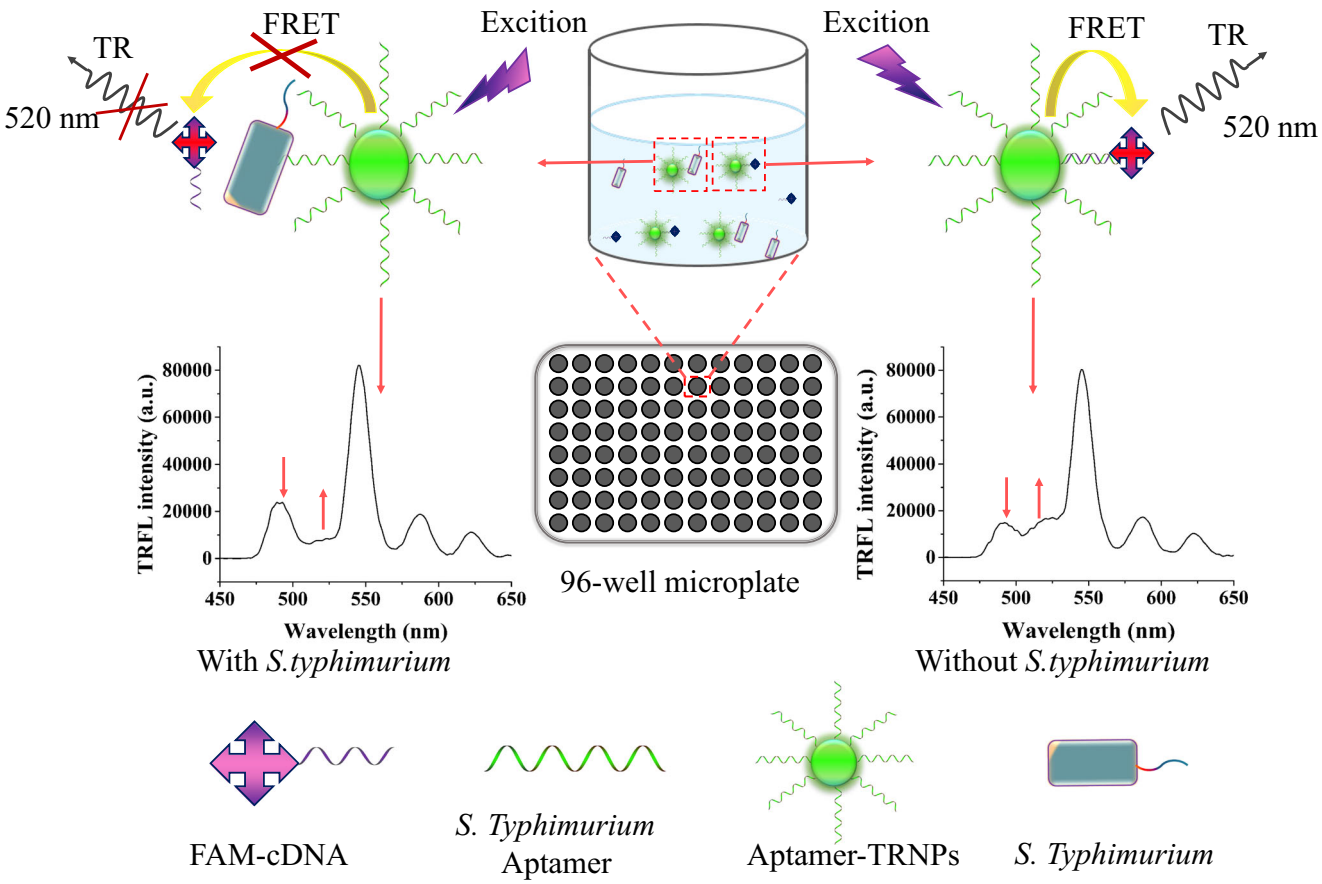
Procedures for detection of S. Typhimurium

To each well, 100 μL of aptamer functionalized NPs (apt-NPs) dispersed in PBS solution was added, followed by the addition of the optimized FAM-cDNA. The plate was incubated for 40 min at 37 °C to form the NPs-FAM complex. Then various concentrations of *S. typhimurium* standard solutions were added to the complex (the final volume of each well is 200 μL) and future incubated at 37 °C for 50 min. Finally the plate was subjected to the measurement of TR-FRET spectra on a microplate reader (Synergy H1, BioTeK) upon excitation at 273 nm, where the delay time and gate time were set to be 100 μs and 1 ms, respectively.

Results and discussion

Detection principle

The principle of TR-FRET bioassay of *S. typhimurium* is illustrated in Scheme 1. In brief, avidin modified NPs is linked to biotin-labeled *S. typhimurium* aptamer, through the biotin-avidin affinity reaction. Complementary oligonucleotide



Scheme 1 Schematic illustration of the time-resolved fluorescence resonance energy transfer from aptamer-modified NPs to FAM for the detection of *S. typhimurium*

(cDNA), which is labeled with FAM, is selected as energy acceptor, in view of its broad excitation peak centered at 495 nm matches well with the emission band of Tb^{3+} centered at 489 nm (Fig. 1). Through the hybridization between the aptamer and cDNA, the TR-FRET pair is constructed, where the excitation energy is transferred from the NPs to FAM. The energy transfer from the NPs donor would apparently lengthen the fluorescence lifetime of the acceptor, and the lengthened long-lived fluorescence can be temporally separated from their naturally short-lived fluorescence co-excited under UV excitation.

Since the nonradiative FRET relies heavily on the distance between the donor and the acceptor, in the absence of *S. typhimurium*, the distance between the donor (NPs) and the acceptor (FAM) is in close proximity, the excited state energy from the donor can be transferred, which leads to a reduction in the donor's fluorescence intensity (here after referred to as Tb_{489}) and an increase in the acceptor's emission intensity (here after referred to as FAM_{520}). In the presence of *S. typhimurium,* aptamer preferentially bound to *S. typhimurium* causes the dissociation of some cDNA, thereby liberating some FAM-cDNA, leading to a decreased fluorescence intensity of FAM_{520} and increased fluorescence intensity of Tb_{489}. The concentration of *S. typhimurium* can be quantified by the ratio of the integrated TRFL intensities of FAM and Tb^{3+}, as denoted by FAM_{520}/Tb_{489}. The TR-FRET detection based on Ln^{3+}-doped inorganic NPs brings together the advantage of background-free signal of the TR technique and the separation-free convenience of homogeneous assay from FRET.

Characterizations of nanoparticles

In this work, amine-functionalized $NaYF_4$: Ce/Tb NPs was synthesized by one-step solvothermal method with AEP as a surfactant and capping agent. Transmission electron microscopy (TEM) image of the NPs (Fig. 2) demonstrates that the NPs is monodisperse, uniform, roughly spherical with a diameter of approximately 30 nm. The cubic structure and phase purity of the NPs was characterized by powder XRD diffraction (Fig. S2). The elemental composition of the $NaYF_4$: Ce/Tb NPs was determined by X-ray spectroscopy (EDS) (Fig. S3). To explore the potential application in TR-FRET detection, we measured the fluorescence lifetimes for Tb^{3+} doped $NaYF_4$ NPs. The decay curves (Fig. S4) fit well to a single-exponential function, when monitoring the characteristic emissions at 544 nm. The fluorescence lifetime is determined to be 4.71 ms for the NPs samples.

Characterizations of nanoparticles conjugated to aptamer

UV-vis and FT-IR were used to characterize the conjugation of NPs with avidin and aptamer (data shown in Fig. S5, S6, S7 in the supporting information). The strong absorbance of avidin before conjugation to NPs can be seen at 280 nm (Fig. S5), and the absorbance of aptamer is at 260 nm (Fig. S7). After incubation, the absorbance of avidin and aptamer in supernatant became weaker at 280 nm (Fig. S5) and 260 nm (Fig. S7), which prove the successful preparation of aptamer bioprobes using an avidin-biotin system.

Control experiments

The TR-FRET relies heavily on the distance between NPs and FAM. In control experiments in which non aptamer labeled NPs was used in place of apt-NPs, under otherwise identical conditions, no FRET occurred (Fig. S8). The hybridization between aptamer and cDNA was directly conformed by confocal laser scanning microscopy. After apt-NPs incubated with FAM-cDNA for 40 min, the NPs-FAM pair was constructed.

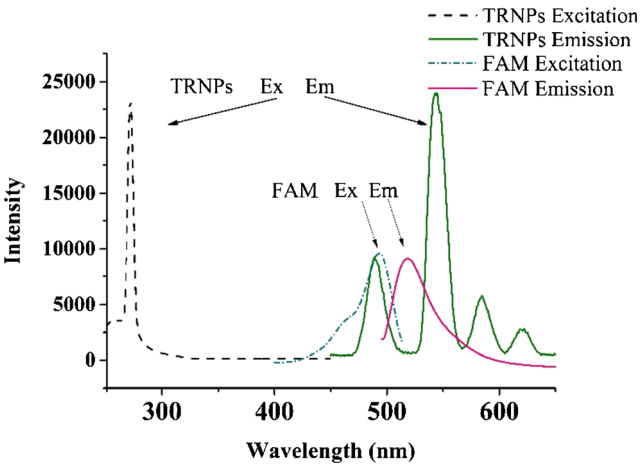


Fig. 1 Excitation (black dashed line) and emission (green solid line) spectra of NPs; excitation (light blue dashed line) and emission (pink solid line) spectra of FAM

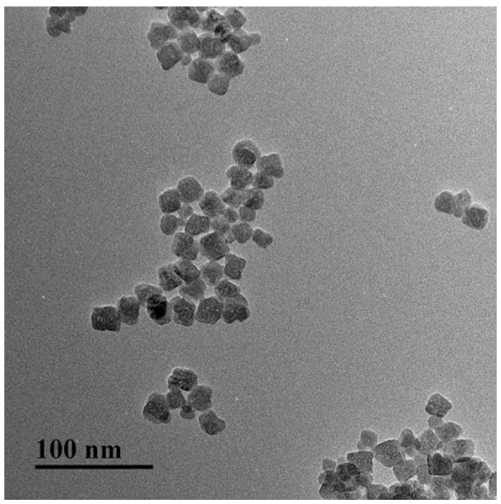


Fig. 2 The TEM image of $NaYF_4$: Ce/Tb NPs

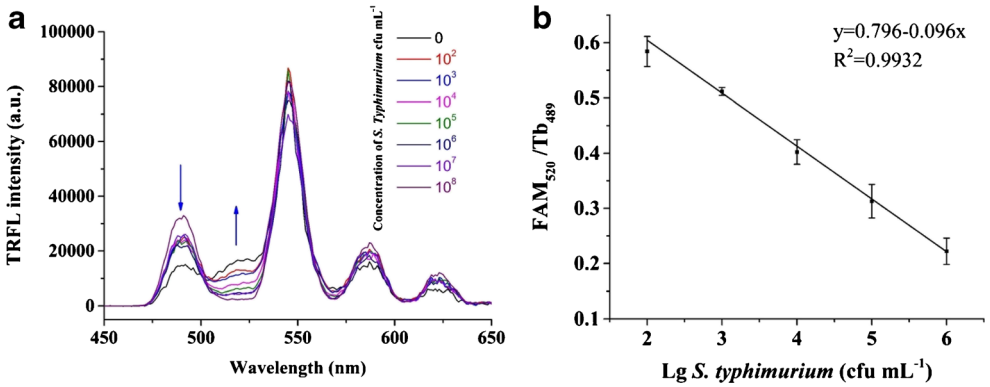


Fig. 3 Typical recording output for the detection of different concentrations of *S. typhimurium* by this method (**a**), standard curve of the integrated TRFL intensity ratio FAM_{520}/Tb_{489} versus the concentration of *S. typhimurium* (**b**)

Apt-NPs shows bright green fluorescence after incubation with FAM-cDNA (Fig. S9).

Optimization of the method

The following parameters were optimized: (a) Concentration of FAM-cDNA; (b) Hybridization time between apt-NPs and FAM-cDNA; (c) Incubation time on FRET between apt-NPs and FAM after adding *S. typhimurium*. Respective data and figures are given in the Electronic Supplementary Material. The following experimental conditions were found to give best results: (a) FAM-cDNA concentration of 400 nM (Fig. S10); (b) Hybridization time of 40 min between apt-NPs and FAM-cDNA (Fig. S11); (c) Incubation time of 50 min after introducing *S. typhimurium* into the system (Fig. S12).

TR-FRET analysis of S. Typhimurium

We developed a TR-FRET biosensing platform for bacteria detection. Determination of *S. typhimurium* was performed in PBS buffer under optimal conditions. The integrated TRFL intensity ratio FAM_{520}/Tb_{489} from the observed TRFL spectra (Fig. 3a) is used to quantify the concentration of *S. typhimurium*. As shown in Fig. 3a, FAM_{520}/Tb_{489} is maximum without *S. typhimurium,* in the presence of *S. typhimurium*, the aptamer preferentially bound to bacteria and causes the dissociation of some FAM-cDNA from apt-NPs with a gradual decrease of the TR-FRET signal (FAM_{520}/Tb_{489}). Under optimal conditions, good linear relationship ($y = 0.796–0.096\times$, $R^2 = 0.9932$) between FAM_{520}/Tb_{489} and the concentration of *S. typhimurium* is observed from 10^2 and 10^6 cfu mL^{-1} with a limit detection (LOD) of 25 cfu mL^{-1} (Fig. 3b).

For comparative purposes, the linear ranges and LODs of several aptasensor for detecting *S. typhimurium* are summarized in Table 1. This method had a relatively low LOD compared with other methods. This benefited from the long fluorescence lifetime of NPs, which greatly improved the signal-to-background ratio. Moreover, comparing this novel method with our previous work [29], which needs magnetic separation, several steps of washing and reagent incubations, this homogeneous TR-FRET assay is more convenient and only required inexpensive equipment. It is therefore better than most of current existing methods.

Specificity assay

To assess the specificity of the TR-FRET-based aptasensor for *S. typhimurium,* the influences of other pathogetic bacteria including *Staphylococcus aureus, E.coli*, *Bacillus cereus* and

Table 1 Comparison of the linear ranges and LODs of several aptasensors for *S. Typhimurium* detection

Foodborne pathogens	Aptasensor principle	Linger range (cfu mL^{-1})	LOD (cfu mL^{-1})	Ref
S. Typhimurium	**TRFL**: Time-resolved nanoparticles as signal probes and magnetic nanoparticles as the capture probes	10^2–10^5	15	[29]
	FRET: FAM as energy donor and GO as quencher	10^3–10^8	100	[30]
	FRET: Quantum-dots (QDs) as energy donor and carbon nanoparticles (CNPs) as acceptor	50–10^6	35	[31]
	FRET: FAM as energy donor and GO as quencher	10^2–10^5	300	[32]
	Surface plasmon resonance (SPR) biosensor	10^2–10^7	60	[33]
	Electrochemical immobilization	75–7.5 × 10^5	25	[34]
	TR-FRET: Time-resolved nanoparticles as energy donor and FAM as aceptor	10^2–10^6	25	This work

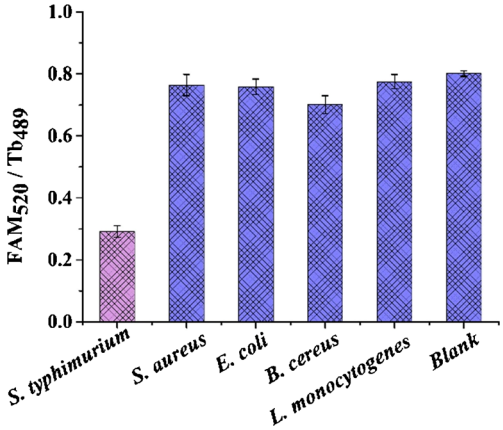


Fig. 4 Specificity assay of this method for detecting various foodborne pathogens

Listeria monocytogenes were examined under the same conditions, and the concentrations of all bacteria were 10^5 cfu mL^{-1}. As shown in Fig. 4, the result indicates that only *S. typhimurium* caused obvious change in the TR-FRET signal (FAM_{520}/Tb_{489}), however, the coexisting species do not exhibit any significant changes and are analogous to the TR-FRET signals of the blank. This designed aptamer biosensor using TR-FRET strategy is highly selective because the target bacteria only perfectly recognize and bind with specific NPs-labeled aptamer, whereas other coexisting species show weak binding with aptamer. The result clearly demonstrates that the designed TR-FRET based aptamer biosensor had good specificity for the detection of *S. typhimurium*.

Application of the new method in chicken meat and egg sample

To demonstrate the feasibility of the practical application of this method, we detected *S. typhimurium* in chicken meat and egg respectively. The pre-treated samples were spiked with between 10^2 and 10^4 cfu mL^{-1} *S. typhimurium,* and then analyzed. As shown in Table 2, the results obtained by this method are close to those obtained by the plate counting method, and no significant differences between the compared methods are observed. The application performance indicates that the TR-FRET assay with aptamer has the ability to efficiently detect and quantify bacteria in real samples.

Table 2 Comparison of egg and chicken meat sample results obtained by the proposed method and by classical plate counting methods

Egg sample	Plate counting (cfu/mL)	This method (cfu/mL)
NO.1	$(1.2 \pm 0.08) \times 10^2$	$(1.1 \pm 0.18) \times 10^2$
NO.2	$(0.9 \pm 0.02) \times 10^3$	$(1.0 \pm 0.11) \times 10^3$
NO.3	$(1.0 \pm 0.13) \times 10^4$	$(1.1 \pm 0.17) \times 10^4$
Chicken meat sample		
NO.1	$(1.1 \pm 0.09) \times 10^2$	$(1.0 \pm 0.13) \times 10^2$
NO.2	$(1.2 \pm 0.11) \times 10^3$	$(1.1 \pm 0.15) \times 10^3$
NO.3	$(1.1 \pm 0.12) \times 10^4$	$(1.1 \pm 0.18) \times 10^4$

Conclusions

We have developed an aptasensor via TR-FRET between NPs and fluorescent dye for rapid, sensitive and specific detection of *S. typhimurium.* The application of time-resolved fluorescence signal effectively removes the autofluorescence noise of biomolecules and debris (impurities) in samples. The use of the FRET system provides an efficient method for *S. typhimurium* detection in one single step. In addition, the aptamer exploited in our approach is easily available and more stable than commonly used antibodies, also the highly specific to the target bacteria demonstrated the potential to detect other bacteria by substituting suitable aptamers. We envision that the methods and principles presented here can be potentially used in pathogenic bacteria detection in food samples.

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Compliance with ethical standards The authors declare that they have no competing interests.

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