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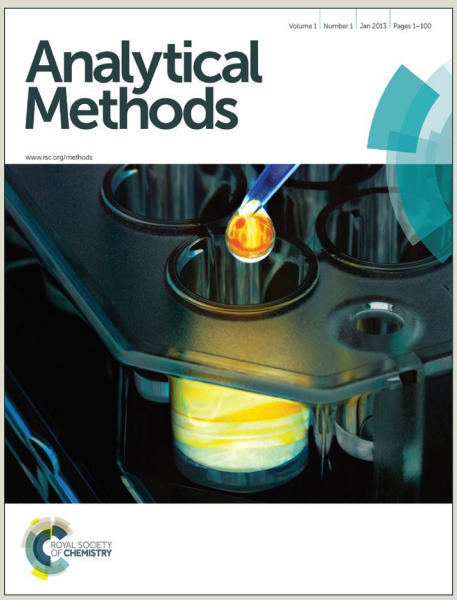

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ARTICLE

Aptasensor based on fluorescence resonance energy transfer for multiplexed pathogenic bacteria determination

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Techniques that are simple and suitable for the identification of multiple pathogenic bacteria are vital to ensure food safety. It has been a critical demand for techniques that can recognize numerous bacterial pathogens simultaneously in a single assay. In this work, a multiplexed assay on an aptamer-based platform is presented using multicolor dyes as donors and carbon nanoparticles (CNPs) as a sole acceptor in fluorescence resonance energy transfer (FRET) for simultaneous detection of multiple bacteria. The multicolor fluorescence probes fluorescein amidite (FAM)-apt 1, cyanine dye 3 (Cy3)-apt 2 and 6-carboxy-X-rhodamine (ROX)-apt 3 were used for recognizing specific targets, which bound to the surface of CNPs through π-π stacking interaction. As a result, the energy donors and acceptor were taken into close proximity, leading to the quenching of the fluorescence of the multicolor dyes. In the presence of the three bacteria, the aptamers preferred to bind to their corresponding pathogens, which led to changes in the formation of the aptamers, and thus, the aptamer-labeled dye dissociated from the CNPs surface. The restored fluorescence intensity of FAM, Cy3 and ROX was related to the concentration of the three bacteria. The applicability of the bioassay in real food samples was also investigated, and the results were consistent with the experimental results obtained from plate-counting methods.

Introduction

Foodborne pathogens can contaminate food at every step of food production, storage, and distribution. *S. typhimurium*, *V. parahaemolyticus*, and *S. aureus* account for a large number of food-borne illnesses worldwide. Around 93.8 million gastroenteritis-related infections were caused by Salmonella, leading to 155,000 deaths each year around the world [1]. From 2003 to 2008, 2795 foodborne disease outbreaks were reported in China. Of the 2227 outbreaks with a known etiologic agent, *V. parahaemolyticus* was confirmed in 322 (14%), resulting in 9041 illnesses, 3948 hospitalizations, and no deaths [2]. In 2011, staphylococcal enterotoxins caused 345 food-borne outbreaks in the EU [3]. The detection of pathogenic bacteria is vital for clinical diagnosis, the prevention and control of infectious diseases and public health. Moreover, multiple bacterial pathogens may exist simultaneously in the same food sample at different concentrations but typically occur at low levels [4,5]. Consequently, there has been a critical demand for techniques that can recognize numerous bacterial pathogens simultaneously in a single assay (multiplexing), thereby providing an early warning of bacterial contamination. The existing simultaneous detection methods for multiple pathogens include multiplex polymerase chain reaction (PCR) or real-time PCR detection [6,7] as well as flow cytometry [8,9]. PCR-based methods, similar to traditional methods, can produce conclusive results but much faster (hours instead of days), and are probably still robust and reliable approaches. However, PCR-based methods are for DNA testing, which are indirect detection methods for pathogenic bacteria. Flow cytometry (FCM) is currently a widely used method for analyzing cell and particle behavior under flow conditions. FCM has become one of the best options for the rapid detection and quantification of a variety of bacteria in environmental, food and clinical samples. However, the sensitive detection of pathogenic bacteria is still a challenge for FCM, as it is hindered by the small size and consequently the low contents of specific cellular constituents of microbial cells [10]. Therefore, it is very necessary to develop a sensitive and simple detection technique for multiple bacterial pathogens.

Fluorescence resonance energy transfer (FRET) is a typical homogeneous assay technique, based on nonradiative energy transfer from energy donors to energy acceptors within close proximity (normally 1-10 nm) via long-range dipole-dipole interactions [11]. The experiment procedure of FRET is simple and convenient without a separation step. However, few studies have used FRET technology for the simultaneous

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detection of various pathogenic bacteria [12]. The current existing FRET methods are most for single pathogenic bacteria detection based on one donor to one acceptor [13,14]. If the goal of simultaneously detecting multiple pathogens is to be achieved multiplexed FRET would be required based on multi-donors to one acceptor. Besides, the recognition mechanism of the existing FRET for pathogenic bacteria detection is based on antigen-antibody immunoreactions (Jung et al., 2010). The preparation of the antibodies via animal immunization is time-consuming (several months), and the antibody may become susceptible to stability or modification issues.

Aptamers, short single-stranded nucleic acids (DNA or RNA), can bind with high affinity and specificity to a wide range of target molecules, such as ions, small organic molecules, and proteins [15-17]. Compared with antibodies, aptamers exhibit a number of advantages. Aptamers can be routinely produced by chemical synthesis, avoiding the use of animals required for antibody production. They are generally more chemically stable, and their binding properties are easier to manipulate. These features hold great promise in therapeutic, diagnostic, and analytical applications [18,19]. A series of aptamers have been created in our lab to target live bacteria [20-22]; and many methods based on aptamers have also been applied to the detection of pathogenic bacteria [9, 23-24] in our previous reports.

The sensitive and simultaneous detection of multiple pathogens in complex food samples has become an important issue in food safety. Therefore, a unique aspect of our work is that we first developed a multiplexed FRET-based aptamer biosensor for the simultaneous detection of various pathogenic bacteria in food. Multicolor dyes act as donors and CNPs as the sole, effective acceptor in the multiplexed FRET strategy. In combination with the use of aptamers as the recognition elements, our developed method demonstrated rapid, sensitive and specific advantages over other methods for the simultaneous detection of various pathogenic bacteria.

Experimental

Materials and apparatus

The *V. parahaemolyticus* aptamer and *S. typhimurium* aptamer used in our research were prepared in our laboratory using previously reported methods [20,21]. *S. aureus* aptamer was reported by Cao et al [25]. Bacteria aptamers were synthesized by the Shanghai Sangon Biological Science & Technology Company (Shanghai, China) and labeled at the 5' end with different dyes: FAM, Cy3 and ROX. The DNA sequence of the *V. parahaemolyticus* aptamer is 5'-FAM-C6-ATAGGAGTCACGACG ACCAGAATCTAAAAATGGGCAAAGAAACAGTGACTCGTTGAGATA CTTATGTGCGTCTACCTCTTGACTAAT-3' (apt 1). The DNA sequence of the *S. aureus* aptamer is 5'-Cy3-C6-GCAATGGTACGGTACTTCCTCGGCACGTTCTCAGTAGCGCTCGCTG GTCATCCCACAGCTACGTCAAAAGTGCACGCTACTTTGCTAA-3' (apt 2). The DNA sequence of the *S. typhimurium* aptamer is 5'-ROX-C6-ATAGGAGTCACGACGACCAGAAAGTAATGCCCGGTAG TTATTCAAAGATGAGTAGGAAAAGATATGTGCGTCTACCTCTTGAC TAAT-3' (apt 3). All reagents were of analytical grade, and buffers were prepared in Millipore water.

Transmission electron microscopy was performed with a JEOL model 2100HR instrument operating at 200 kV accelerating voltage (TEM, JEOL Ltd., Japan). UV-visible absorption spectra were measured using a UV-1800 spectrophotometer (Shimadzu Co., Japan). The fluorescence intensities were measured on an F-7000 fluorescence spectrophotometer (Hitachi Co., Japan). Imaging of the bacterial cells was performed with a ZEISS LSM 710 confocal microscope (Carl ZEISS, Germany). The samples were placed above a 40×objective and excited with the 488 nm line of the argon-ion laser, and emission was detected using a 505-525 nm bandpass filter for FAM signal, a 535-565 nm bandpass filter for the Cy3 signal, and a 610-nm long-pass filter for the ROX signal.

Bacterial strains and culture media

The *V. parahaemolyticus* ATCC 17802, *S. aureus* ATCC 29213, and *S. typhimurium* ATCC 14028 were kindly provided by the Animal, Plant and Food Inspection Centre, Jiangsu Entry-Exit Inspection and Quarantine Bureau (Nanjing, China). All bacteria were grown overnight in a liquid culture (*S. aureus* and *S. typhimurium* were grown in LB media, *V. parahaemolyticus* was grown in alkaline peptone with 3% NaCl (w/v) until an OD_{600} of 0.3 was obtained. Cells were pelletized at 3000 rpm and 4 °C and washed twice in a 1×binding buffer (50 mM Tris-HCl at pH 7.4, 5 mM KCl, 100 mM NaCl, and 1 mM MgCl2) at room temperature. After incubation at 37 °C for 18 h, the bacteria solution was diluted 102~106 times and 100 µL of bacteria diluent was took to coat plate. The colonies on the plates were counted to determine the number of colony-forming units per milliliter (cfu/mL).

Synthesis of carbon particles

Carbon nanoparticles were synthesized using candle soot as the starting material following literature reports [26]. During a typical synthesis, carbon soot (25 mg, collected from a burning candle) was added to 15 mL of 5 M nitric acid in a 25 mL three-necked flask. It was then refluxed for 12 h under magnetic stirring at 100 °C. Next, the black solution was cooled and centrifuged at 3000 rpm for 10 min to separate and remove the unreacted carbon soot. The light brownish-yellow supernatant was collected and mixed with acetone (water/acetone volume ratio of 1:3) and centrifuged at 14 000 rpm for 10 min. The black precipitate was collected and dissolved in 5 mL of water at a concentration calculated as ca. 1.6 mg/mL.

Preparation of real samples

Prior to being used for testing, 5 mL of milk was centrifuged at 7000 rpm for 10 min at 10 °C, and the upper layer of cream was removed. After this fat was removed, the milk was diluted with distilled water in a 1:20 ration before filtering with a disposable syringe filter holder. Fresh salmon were purchased from a local supermarket and transported to the laboratory in containers with ice bags. A 25 g salmon sample was aseptically dipped in 225 mL of alkaline peptone with 3% NaCl (w/v) and

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homogenized for 5 min. The product was allowed to stand for 30 min to precipitate macroaggregates and seston. The supernatant was subsequently filtered through a 0.45 μm filtration membrane.

Fluorescence assays

Since FRET is a homogeneous assay technique, the experiment procedure is simple and convenient. For multiplexed detection, 350 μL of 1×binding buffer containing three dye-labeled aptamers (dye-apts) and 15 μL of 1.6 mg/mL CNPs (final concentration of 0.06 mg/mL) was first incubated for 70 min at room temperature. Next, a mixed solution of three different bacteria targets (40 μL) was added to the binding buffer, and the solution was further incubated for 1.5 h at room temperature. The fluorescence signals were measured at three wavelengths, 520 nm (FAM), 564 nm (Cy3) and 608 nm (ROX), by excitation at a single wavelength (495 nm). Slits for both the excitation and the emission were set at 5 nm for all dyes.

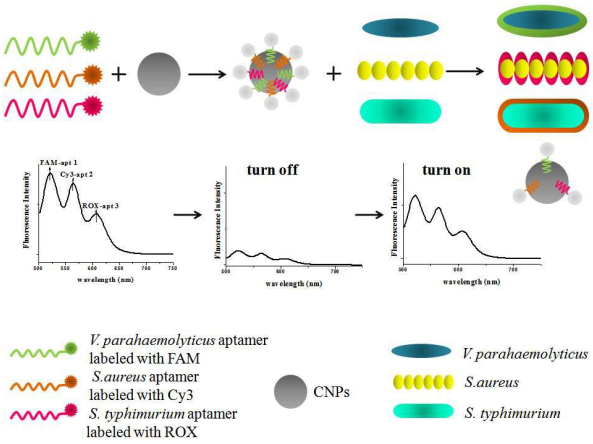
Statistics assay

F-F_0 was plotted as the sensor signal, where F_0 is the background fluorescence which is in the absence of target bacteria. F is the fluorescence of the three dyes in the presence of the target bacteria. All experiments were repeated three times, each sample solution was measured five times, and the standard deviation was plotted as the error bars.

Results and discussion

Principle of multiplexed FRET aptasensor for pathogenic bacteria

Scheme 1 demonstrates the principle of the aptamer biosensor based on FRET for the multiplexed detection of pathogenic bacteria. In multicolor mode, three different aptamers (apt 1, apt 2 and apt 3) labeled at their 5' terminus with different fluorescence dyes (FAM, Cy 3 and ROX) acting as signaling probes are first adsorbed onto CNPs by means of π-π stacking interactions between the nucleotide bases and the CNPs [27]. The π-π stacking interaction between single-stranded DNA and CNPs is an intriguing property of CNPs as an energy acceptor in FRET assays, which omits the labeling of probes and facilitates experimental procedures. The π-rich electronic structure of CNPs, which results from sp^2 orbital hybridization, endows the carbon material with strong fluorescence quenching power as a result of electron transfer from excited donors to the π orbital of carbon atoms [28]. Meanwhile, such a π-rich structure also enables the easy assembly of biomolecules containing an aromatic motif on the surface of CNPs via π-π stacking interaction. This adsorption process brings the reference dyes FAM, Cy3 and ROX into the close proximity of the CNPs, which results in significant fluorescence quenching due to the energy transfer effect. The common absorption spectrum of a typical CNPs sample spans a wide range of wavelengths (approximately 300-700 nm), significantly overlapping the photoluminescence spectra of various fluorophores. This allows FRET to occur. In the presence of specific targets, the aptamer bound to the target with high affinity and specificity and formed a stable dyes-aptamer-target complex.



Scheme 1 Schematic illustration of the multiplexed fluorescence resonance energy transfer from aptamer-modified dyes to carbon nanoparticles for the simultaneous detection of various pathogenic bacteria.

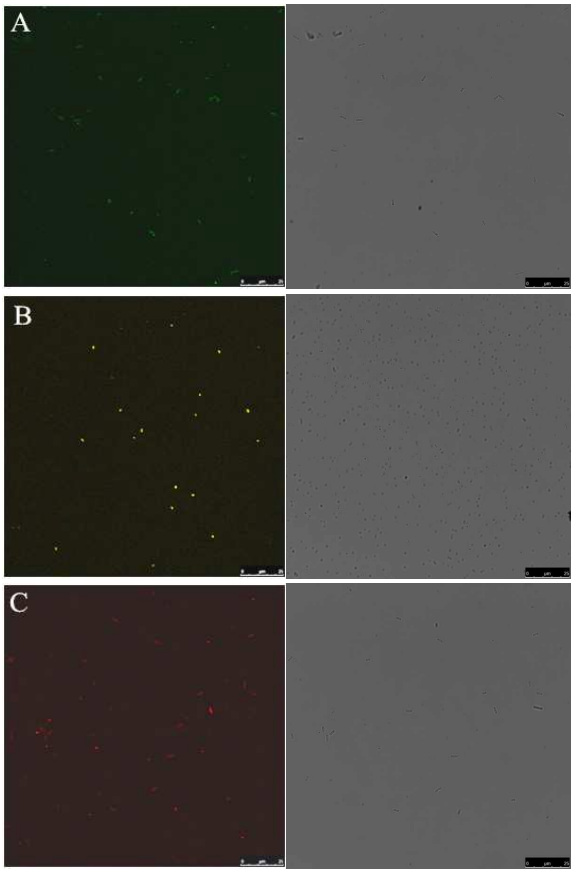


Fig. 1 Fluorescence and bright field images of bactrial cells: A) *V. parahaemolyticus* incubated with FAM-apt 1, showing in green; B) *S. aureus* incubated with Cy3-apt 2, showing in orange; and *S.typhimurium* incubated with ROX-apt 3, showing in red (Fig. 1C).

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The high binding of the aptamers to the target cells were directly confirmed by confocal laser scanning microscopy (Fig. 1). *V. parahaemolyticus* showed bright green fluorescence after incubating with FAM-apt 1 (Fig. 1A), *S. aureus* showed bright orange fluorescence after incubating with Cy3-apt 2 (Fig. 1B), and *S.typhimurium* showed bright red fluorescence after incubating with ROX-apt 3 (Fig. 1C). This complexation leads to changes in the formation of the aptamers and thereby released from the CNPs substrate, resulting in the fluorescence recovery. By monitoring the increase in the fluorescence intensities of the respective dyes, multiple pathogenic bacteria targets were simultaneously detected.

Characterization of CNPs

The carbon materials we prepared were spherical nanoparticles (Fig. 2A). The absorption spectrum of CNPs (Fig. 2B) spans a wide range of wavelengths (approximately 300-700 nm), overlapping the fluorescence spectra of FAM, Cy3 and ROX, in good accordance with the literature results (Li et al., 2011). This remarkable property is responsible for CNPs' crucial role in the system of multiplexed FRET.

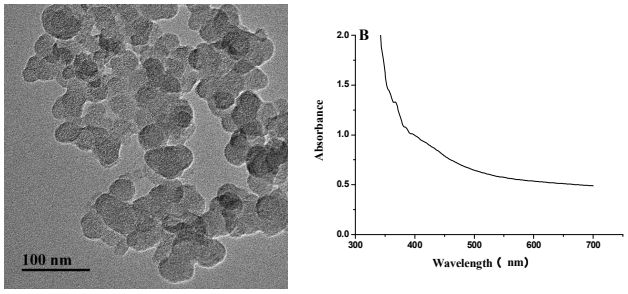

Fig. 2 (A) TEM image of carbon nanoparticles. (B) UV-vis absorption spectrum of the as-prepared carbon nanoparticles with a concentration of 0.04 mg/mL.

Effect of the concentration of the three types of fluorescence dye labelled aptamers

The three probes FAM-apt 1, Cy3-apt 2 and ROX-apt 3 were used in our developed method, where FAM, Cy3 and ROX dyes, of which the emission wavelength is 520, 564, and 608 nm, respectively, were used as the signals. The most suitable excitation wavelength is 495 nm for FAM, 550 nm for Cy3, and 580 nm for ROX. However, the three fluorescence dyes were required to exhibit multicolor emission under one single wavelength excitation for multiplexed bioassays. The excitation wavelength was thus set at 495 nm. However, Cy3 and ROX dye exhibit a relatively weak fluorescence signal under excitation at 495 nm. For this reason, the mixing ratio of these three probes was optimized so that they could exhibit a relatively high and balanced fluorescence signal. As shown in Fig. 3, by varying the relative concentrations of each probe, the three probe emission signatures can be adjusted and exhibit different appropriate colors under excitation at 495 nm at a mixing ratio of 1:3:5 (1=100 nM). Therefore, 100 nM FAM-apt 1, 300 nM Cy3-apt 2 and 500 nM ROX-apt 3 were used in the present work.

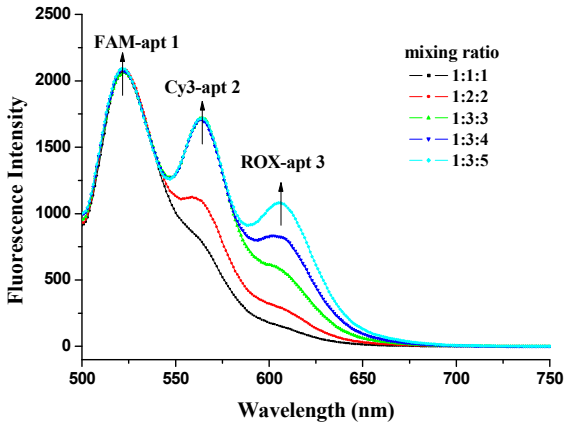

Fig. 3 Fluorescence emission spectra of the mixture of FAM-apt 1, Cy3-apt 2 and ROX-apt 3 with different mixing ratio (1:100 nM). Ex=495 nm.

FRET between dye-Labeled aptamer and CNPs

The fluorescence of the aptamers labeled with FAM、Cy3 and ROX dyes can be efficiently quenched by CNPs due to FRET between the three fluorescence dyes and CNPs. Fig. 4 shows the fluorescence quenching of FAM-apt 1, Cy3-apt 2 and ROX-apt 3 at various concentrations of CNPs. The fluorescence intensity decreased rapidly when the CNPs were added into the mixture. Moreover, the fluorescence intensity decreased with increasing concentration of CNPs. Over 80% of the fluorescence was quenched by the addition of 0.06 mg/mL CNPs. The high quenching efficiency is considered to be a direct consequence of the noncovalent binding of the aptamer on the CNPs surface and the energy transfer from the dyes to the CNPs. This strong binding between the aptamers and CNPs causes the labeled fluorophores to approach to the CNPs, inducing high efficiency energy transfer between the dyes and CNPs. Because CNPs is a very good quencher of a wide variety of dyes, it is relatively easy to construct a multiplex FRET sensing system using CNPs as the quencher.

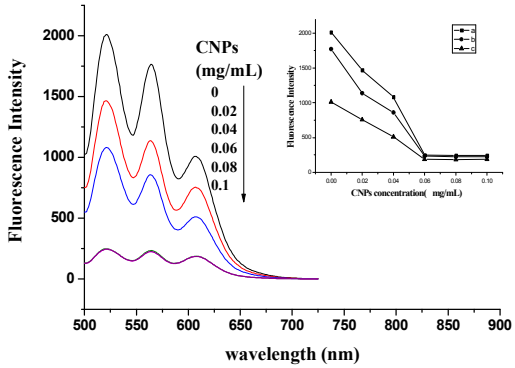

Fig. 4 Fluorescence quenching of aptamers labeled with dye with varying amounts of CNPs. Inset: fluorescence intensity (a: FAM-apt 1,

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b: Cy3-apt 2, c: ROX-apt 3) versus CNPs concentration (0, 0.02, 0.04, 0.06, 0.08 and 0.1 mg/mL).

Effect of incubation time for fluorescence quenching and restoration

Fig. 5A shows the fluorescence quenching of FAM-apt 1, Cy3-apt 2 and ROX-apt 3 in the presence of CNPs as a function of incubation time (curve a, b and c). As seen, the fluorescence intensity exhibits an immediate reduction in the first 40 min for all dye-labeled aptamers, and a sustained decrease over a period of 60 min. The fluorescence intensity of all dyes reaches a minimum and ceases to change after 70 min. Therefore, the incubation time of the three dye-apts and CNPs was set at 70 min. Subsequently, in the presence of the specific targets, with the formation and release of the aptamers from CNPs, the fluorescence intensity of FAM-apt 1 exhibits a rapid increase in the first 40 min, followed by a slow increase over a period of 60 min (Fig. 5B, curve a'). However, the best fluorescence restoration of Cy3 and ROX was achieved after 90 min of incubation (curve b' and c'). Therefore, an incubation time of 90 min for targets and CNPs-dye-apts was selected for further study.

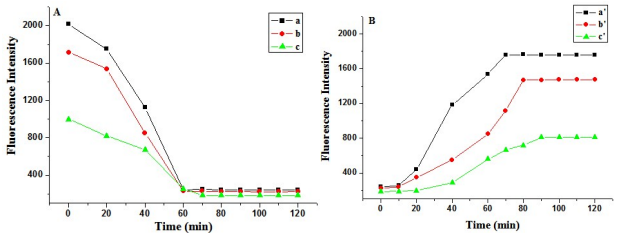

Fig. 5 Effect of incubation time on the fluorescence quenching of FAM-apt 1 (a), Cy3-apt 2 (b), ROX-apt 3 (c) and fluorescence restoration of FAM-apt 1 (a'), Cy3-apt 2 (b'), ROX-apt 3 (c') in the presence of target. The three bacteria concentration were all 10^4 cfu/mL.

Multiplexed FRET analysis of target bacteria

We developed a fluorescent biosensing platform for multiplexed bacteria detection using a CNPs FRET aptasensor. As shown in Fig. 6A, the fluorescence intensity of the mixture of FAM-apt 1, Cy3-apt 2 and ROX-apt 3 was maximum (curve a). The fluorescence intensity decreased in the addition of CNPs into the mixture (curve g). With the increase of concentrations of specific bacteria, the fluorescence intensity increased gradually (curve b~f). The relative fluorescence intensity is plotted against bacteria concentration in Fig. 6B. Under the optimal conditions, good linearity relationships between the relative fluorescence intensity and the concentration of *V. parahaemolyticus*, *S. aureus*, and *S. typhimurium* was observed from 10^2 to 10^6 cfu/mL with the limits of detection (LOD) of 25, 50, and 50 cfu/mL, respectively. As shown in Table S1, all of the calibration curves were observed over the tested range. The recovered relative fluorescence has good reproducibility with a standard deviation of usually less than 5%.

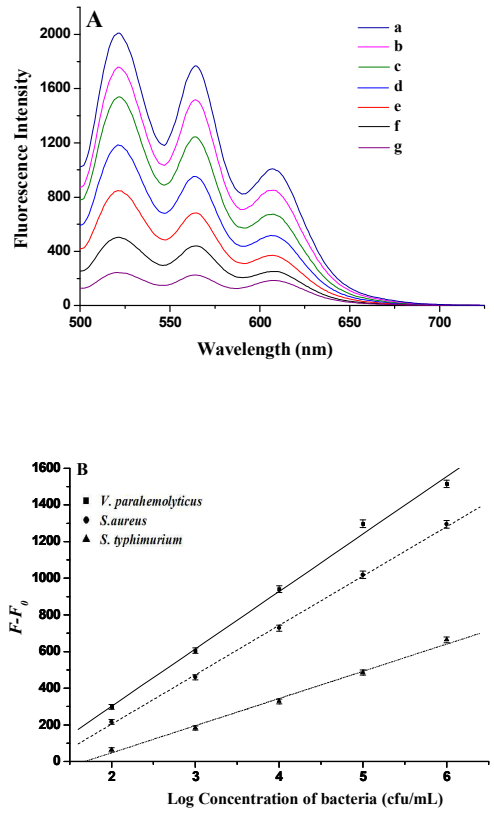

Fig. 6 (A) Typical recording output for the simultaneous detection of different concentrations of three bacteria by the developed method. a: mixture of dyes-apts; b~g: concentration of the three bacteria 10^6, 10^5,10^4,10^3,10^2,0 cfu/mL. (B) Standard curve of the related fluorescence intensity ($F-F_0$) versus the concentrations of three bacteria.

Specificity evaluation

The designed aptamer biosensor using a FRET strategy was highly selective because each target bacteria only perfectly recognizes and binds with the specific dye-labeled aptamer, leading to an increase in the fluorescence of the corresponding dye. To assess the specificity of the multiplexed FRET-based aptamer biosensor for *V. parahaemolyticus*, *S. aureus* and *S. typhimurium*, the influences of other pathogenic bacteria including *Shigella dysenteriae*, *Listeria monocytogenes*, *Cronobacter sakazakii*, *Streptococcus pyogenes* and *Escherichia coli* were examined in the mixture of CNPs-dye-apts, respectively. The concentrations of the tested bacteria were ten-fold higher than that of the target bacteria. As shown in Fig. 7, none of these bacteria caused obvious changes in the fluorescence, while a significant fluorescence increase was observed for the target bacteria. These results clearly demonstrate that the designed multiplexed FRET-based aptamer biosensor had good specificity for the detection of *V. parahaemolyticus*, *S. aureus* and *S. typhimurium*.

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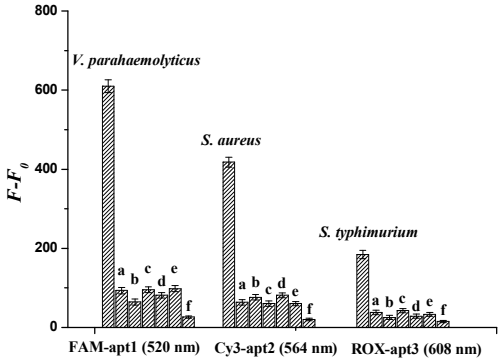

Fig. 7 Selectivity evaluation of the proposed method for V. parahaemolyticus, S. aureus, and S. typhimurium (10^3 cfu/mL) against other bacteria (10^4 cfu/mL). a~f: *S. dysenteriae*, *L. monocytogenes*, *C. sakazakii*, *S. pyogenes*, *E. coli*, and blank, respectively.

Analytical application

To demonstrate the feasibility of the practical application of the proposed method, we detected the target pathogenic bacteria in real samples, i.e., milk and salmon. The pre-treated samples were spiked with between 1×10^3 and 1×10^5 cfu/mL *V. parahaemolyticus*, *S. aureus*, and *S. typhimurium*, and then analyzed. As shown in Table S2, the results obtained by the developed method were close to those obtained by the plate-counting method. There was no significant difference between the methods that were compared. The application performance clearly demonstrated that our multiplex FRET assay with aptamers has the ability to efficiently detect and quantify bacteria cells in real samples.

Conclusions

In this study, we have constructed a new aptamer biosensor based on multiplexed fluorescence resonance energy transfer from multicolor fluorescence dyes to carbon nanoparticles for the simultaneous detection of V. parahaemolyticus, S. aureus, and S. typhimurium. The multiplexed FRET system was first presented and used for pathogenic bacteria determination. Due to the strong fluorescence quenching ability and good biocompatibility of carbon nanoparticles, the multicolor dye-CNPs system was a competitive energy donor-acceptor pair. In addition, the system shows high specificity, as a result of the properties of the aptamers. Therefore, these aptamers offer us a new approach to the fabrication of a convenient, sensitive, specific, and stable platform for bioassays. Furthermore, with the capability of producing specific aptamers for any type of bacteria, we believe that this methodology can be developed for the multiplexed detection of any type of pathogen.

Acknowledgements

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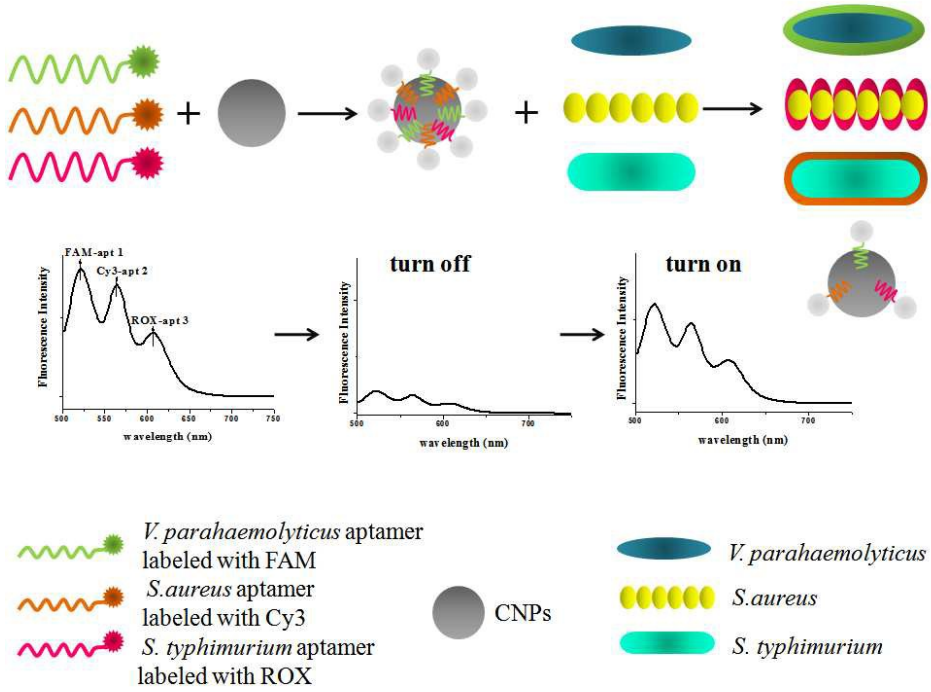
Graphical abstract



A multiplexed FRET-based aptasensor was developed for the simultaneous bacteria detection using multicolor dyes as donors and CNPs as sole acceptor.

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