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Detection of Broad Spectrum Bacteria Using a FITC – Lysozyme and Positively Charged AuNPs Constructed FRET Platform

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Abstract：This manuscript demonstrated a universal platform for bacteria assay by using a fluorescein isothiocyanate-labeled lysozyme（FITC – Lys）and polyethylenimine-modified positively charged gold nanoparticles（PEI – AuNPs）constructed fluorescence resonance energy transfer（FRET）sensor based on its electrostatic binding with bacteria. In the presence of the bacteria，the energy donor of positively charged FITC – Lys bond with the bacteria through both the recognition to peptidoglycan of cell wall and electrostatic interaction while the energy acceptor of positively charged PEI – AuNPs bond with the bacteria based on the electrostatic interaction，which decreases the distance between energy donor and acceptor and then causes fluorescence quenching，showing FRET “on” signal readout mode with the low fluorescence intensity. PEI – AuNPs were synthesized by hydrothermal method and characterized by ultraviolet spectrum，transmission electron microscope，and laser particle size analyzer. The results shown that PEI – AuNPs with diameter about 9.6 nm had maximum ultraviolet absorption at 526.5 nm，and they were positively charged. The method developed in this study could detect 10 different kinds of bacteria such as *Staphylococcus aureus*，*Streptococcus pneumoniae*，*Enterococcus faecalis*，*Staphylococcus epidermidis*，*Listeria monocytogenes*，*Escherichia coli*，*Salmonella typhimurium*，*Salmonella paratyphoid A*，*Vibrio parahaemolyticus*，and *Pseudomonas aeruginosa*. Then，the interference of possible interfering components in actual samples（taking milk and juice as examples）on the detection was further discussed. The results showed that under our experimental conditions，the interfering components such as proteins，ions，and others in the samples would not interfere with the analysis results after ten times dilution. This strategy showed feasibility as a universal assay platform for broad spectrum bacteria，which is interesting for the control of pathogenic bacteria.

Key words：bacteria；lysozyme；gold nanoparticle；FRET

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基于异硫氰酸荧光素标记溶菌酶和荷正电纳米金构建荧光共振能量转移平台检测广谱细菌

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摘 要：通过异硫氰酸荧光素标记的溶菌酶（FITC – Lys）和聚乙烯亚胺修饰的荷正电纳米金粒子（PEI – AuNPs）与细菌结合，构建了一个基于荧光共振能量转移（FRET）的平台用于广谱细菌检测。待检样本中存在细菌时，荷正电的FITC – Lys（能量供体）通过与细胞壁肽聚糖的识别作用和静电作用与细菌结合，荷正电的PEI – AuNPs（能量受体）也通过静电作用与细菌结合，使能量供体和受体之间的距离缩短，荧光猝灭，显示出低荧光强度的FRET“开”的信号读出模式。采用水热法合成了PEI – AuNPs，通过紫外光谱、透射电镜、激光粒度仪等对PEI – AuNPs进行了表征。结果表明，PEI – AuNPs在526.5 nm处有最大紫外吸收，直径约为9.6 nm，荷正电。该方法可对10种细菌（如金黄色葡萄球菌、肺炎链球菌、粪肠球菌、表皮葡萄球菌、李斯特

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菌、大肠杆菌、鼠伤寒沙门氏菌、甲型副伤寒沙门氏菌、副溶血弧菌和铜绿假单胞菌)进行检测。考察了实际样本(以牛奶和果汁为例)中可能的干扰组分对检测的干扰情况。结果表明,将样本稀释10倍后,样本中蛋白、离子等不会对分析结果产生干扰。该研究构建的广谱细菌检测方法,对于致病菌的防控具有积极意义。

关键词:细菌;溶菌酶;纳米金颗粒;荧光共振能量转移

Due to the lack of on-site effective supervision of food safety in the processing environment and processing procedures[1], pathogens contamination causing by *S. aureus*, *E. coli*, *L. monocytogenes*, *Salmonella*, *V. parahaemolyticus*, *Bacillus cereus*(*B. cereus*) in milk, meat, poultry and egg products[2-4] can cause gastrointestinal infections[5], purulent skin infections[6], pneumonia[7], meningitis[8], sepsis[9] and many other diseases seriously threaten food safety and public health[10-20]. There are about 23 000 deaths caused by antibiotic-resistant bacterial infection in the United States every year, and the death rate caused by pathogens is increasing yearly[21]. It is estimated that there will be about 10 million deaths every year in the world by 2050[22]. Due to the great harm caused by bacteria to health and the complex of contaminated bacteria, it is particularly important to develop universal method for detecting bacteria.

Plate counting is classical method of identifying bacteria, but it is limited by long incubation time (24-48 h), the requirement of absolutely clean experimental platform, and tedious operation step[23]. Therefore, some rapid detection methods have been developed, such as polymerase chain reaction(PCR), but is limited by professional instruments for DNA extraction and false positive[24-26]. Immunological detection method is limited by the great difference between antibody batches and poor stability[27-28]. Electrochemical detection and colorimetric detection are susceptible to external interference[29-30]. Flow cytometry and gene chip method need large instruments and are limited by their specialty[31-33]. What's more, current methods mostly focus on the detection of a specific kind of bacteria, a universal method is needed due to the wide distribution of bacteria.

Fluorescence resonance energy transfer(FRET) is a non-radiative energy transfer process through long-range dipole-dipole interaction between donor-acceptor pair[34]. FRET-based strategies have achieved sensitive and rapid detection of various targets such as organophosphate pesticides[35-37], veterinary drugs[36-37], mycotoxins[37-39], heavy metal ions[39-40], biomarker[41-42], virus[43] and pathogenic bacteria[44] with high accuracy.

Lysozyme(Lys) is a protein that can specifically hydrolyze the β-1,4 glycosidic bond between N-acetyl muramic acid and N-acetyl glucosamine in peptidoglycan of bacterial cell wall[45]. In previous studies, fluorescein isothiocyanate-labeled lysozyme(FITC-Lys) was used to detect Gram-positive bacteria(G^+) bacteria based on the recognition of lysozyme to the peptidoglycan of bacteria by recording the fluorescence intensity of supernatant or precipitate after centrifugation[46-47]. However, this method has the limitation of bad accuracy and only detecting some G^+ bacteria. At present, a lot of studies have confirmed that modification of lysozyme can broaden its ability to act on Gram-negative bacteria based on electrostatic binding between the modified lysozyme and Gram-negative bacteria[45,48-51]. Therefore, adjusting pH to make lysozyme positively charged can broaden its antibacterial spectrum.

The emission wavelength of fluorescein isothiocyanate(FITC) overlaps with the absorption spectrum of gold nanoparticles(AuNPs), which is a common donor-acceptor pair[52]. In this study, FITC-Lys and polyethylenimine-modified positively charged gold nanoparticle(PEI-AuNPs) constructed FRET universal platform for broad spectrum bacteria detection based on electrostatic interaction between each other. Specifically, after adjusting pH of the reaction buffer lower to the isoelectric point of FITC-Lys and making the FITC-Lys positively charged, FITC-Lys could bind to all bacteria through both the peptidoglycan recognition and electrostatic interaction with bacteria. Other hand, PEI-AuNPs could bind with bacteria through electrostatic interaction. In the presence of bacteria (no matter of G^+ or G^- bacteria), FITC-Lys and PEI-AuNPs would close each other due to their corresponding binding with bacteria, resulting in fluorescence quenching and showing FRET "on" signal readout mode(Fig.1). The results showed that, based on the FITC-Lys and PEI-AuNPs FRET platform, a series of pathogenic bacteria (no matter of G^+ or G^- bacteria) could be detected simply, showing

universal assay platform for broad spectrum bacteria.

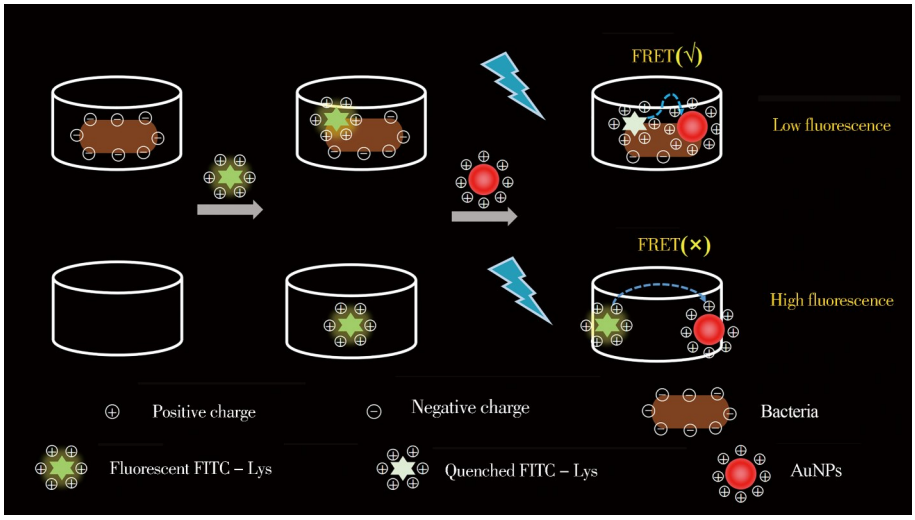

Fig. 1 Illustration for the detection of bacteria based on FITC – Lys and PEI – AuNPs constructed FRET platform

1 Experimental

1.1 Apparatus and reagents

All experimental water was prepared by Milli-Q ultrapure water system (Millipore Company, USA) with a resistance of 18.2 MΩ·cm. Fluorescent spectroscopy was recorded on a fluorescence spectrophotometer (F – 7000, Hitachi). UV – Vis absorption spectra were obtained using an ultraviolet spectrophotometer (UV – 2450, Shimadzu company). The hydrated particle size and ζ-potential were measured using a dynamic light scattering Malvern instrument (NanoZS ZEN3600, United Kingdom). Fluorescence intensity were obtained by multifunctional microplate reader (SpectraMax iD3, Meigu molecule). Morphology was characterized using a transmission electron microscope (TEM) (JEM1200EX, JEOL, Japan).

Chloroauric acid ($HAuCl_4 \cdot 4H_2O$), polyethylenimine (PEI) and casein were provided by Shanghai Macklin Biochemical Co., Ltd. Lysozyme labeled with fluorescein isothiocyanate (FITC – Lys) was obtained from Xi′ an Ruixi Biology Co., Ltd. Fluorescein isothiocyanate (FITC), bovine albumin (BSA) and bovine albumin labeled with fluorescein isothiocyanate (FITC – BSA) were purchased from Beijing Solarbio Science & Technology Co., Ltd. β-Lactoglobulin was provided by Shanghai Yuanye Biology Science and Technology Co., Ltd. Glucose and sucrose were purchased from biofroxx Biotechnology Co., Ltd. Ascorbic acid, calcium chloride, dipotassium hydrogenphosphate, potassium chloride and sodium chloride were obtained from Chongqing Chuandong Chemical Industry Group Co., Ltd. *S. aureus* ATCC 29213, *S. pneumonia* ATCC 10015, *E. faecalis* ATCC 19433, *L. monocytogenes* ATCC19115, *S. typhimurium* ATCC 27853 and *V. parahaemolyticus* ATCC17802 were obtained from China General Microbiological Culture Collection Center. *E. coli* O157: H7 CICC 21530 and *S. paratyphoid A* CICC 21501 were obtained from China Center of Industrial Culture Collection. *S. epidermidis* CCTCC AB 91100 and *P. aeruginosa* CCTCC 93078 were obtained from China Center for Type Culture Collection.

1.2 Synthesis of PEI – AuNPs

PEI – AuNPs were prepared according the previous published work[53]. Specifically, PEI (69.4 mg) dispersed in 9.5 mL of primary water was mixed with 823 μL of 1% $HAuCl_4$, reacting at 65 ℃ for 1 h with vigorous stirring. After removing supernatant by centrifugation at 18 000 r/min for 20 min, the precipitate was dispersed with ultrapure water and stored in a refrigerator at 4 ℃.

1.3 Binding of PEI – AuNPs with bacteria

Bacteria (10^9 cfu/mL) were incubated with PEI – AuNPs (4 nmol/L) at 37 ℃ for 1 h, and then the samples were washed by centrifugating (2 000 r/min, 5 min) for 3 times.

1.4 Detecting bacteria with FITC – Lys and PEI – AuNPs

FITC – Lys(70 μg/mL) was incubated with *S. aureus*, *S. pneumoniae*, *E. faecalis*, *S. epidermidis*, *L. monocytogenes*, *E. coli*, *S. typhimurium*, *S. paratyphoid A*, *V. parahaemolyticus* and *P. aeruginosa* (10^8 cfu/mL) for 30 min, and then the mixture was incubated with PEI – AuNPs/AuNPs(10 nmol/L) for 1 h, following measuring their fluorescence intensity.

2 Results and discussion

In our previous work, the dual-molecule recognition-based strategies were developed to detect bacteria[54-56]. In these strategies, specific antibiotics and aptamer which both could only recognized some special kinds of bacteria were employed as recognition molecules, meaning that different other recognition molecules are required to employed if detecting other types of bacteria. Therefore, a universal detection platform for broad spectrum bacteria assay is needed to explored. In this study, energy donor of FITC – Lys and energy acceptor of PEI – AuNPs were employed to construct FRET universal platform for broad spectrum bacteria detection.

2.1 Potential distribution of different bacteria and protein at pH 4

The isoelectric point of lysozyme is 11.0, which has high positive charge[57]. Bacterial cell walls contain various functional groups, which provide binding sites for solutes in the environment. Therefore, under most pH conditions, bacterial cell walls are often negatively charged and have strong affinity with positively charged substances[58]. As shown in Fig. 2, bacteria of *S. aureus*, *S. pneumoniae*, *E. faecalis*, *S. epidermidis*, *L. monocytogenes*, *E. coli*, *S. typhimurium*, *S. paratyphoid A*, *V. parahaemolyticus*, *P. aeruginosa*, and FITC – BSA were all negatively charged at pH 4, while FITC – Lys was positively charged at this condition.

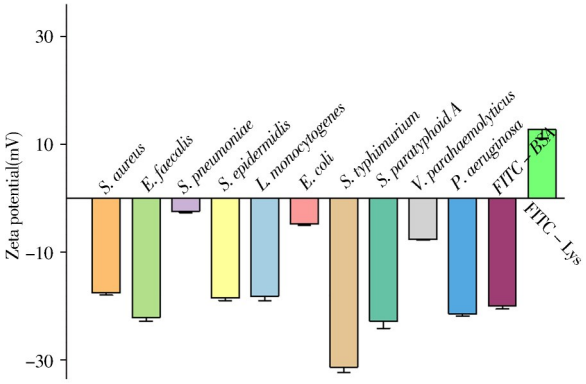

Fig. 2 Zeta potential distribution of different bacteria, FITC – BSA and FITC – Lys

2.2 Characterization of PEI – AuNPs

PEI – AuNPs was synthesized by using PEI as protective agent and reducing agent according to the literature with slightly modification[53]. The PEI – AuNPs has the maximum ultraviolet absorption at 526.5 nm (Fig. 3A). The hydrodynamic size (about 11.22 nm) of PEI – AuNPs measured by dynamic light scattering (Fig. 3B) is consistent with the size about 9.6 nm shown in the TEM image (Fig. 3C). The PEI – AuNPs is positively charged with zeta potential about + 33.7 mV. To verify the binding of bacteria with PEI – AuNPs, the mixture of bacteria with PEI – AuNPs was centrifuged to observe the precipitate after incubation. As shown in Fig.3D, compared with the yellow or white precipitate from the pure bacteria samples, the mixed samples of bacteria and PEI – AuNPs showed red precipitate, which was attribute to the binding of PEI – AuNPs with bacteria.

2.3 Selection of the procedures to detect bacteria

By taking *S. aureus* as a model, we tried to detect the bacteria according the procedure (FITC – Lys and PEI – AuNPs mixed firstly and then co-incubated with *S. aureus*) published in our previous work. Unfortunately, there wasn't any change of the fluorescence intensity. Then another two different detection procedures (PEI – AuNPs and *S. aureus* mixed firstly and then co-incubated with FITC – Lys, FITC – Lys and *S. aureus* mixed firstly and then co-incubated with PEI – AuNPs) were studied. The results showed that, if PEI – AuNPs incubated with *S. aureus* firstly then incubated with FITC – Lys, there also wasn't any change of the fluorescence intensity. Fortunately, when FITC – Lys incubated with *S. aureus* firstly then incubated with PEI – AuNPs, an obvious change of the fluorescence intensity was observed, suggesting that using this procedure might be feasible to detect bacteria. This phenomenon might be attribute to that the electrostatic binding capacity between PEI – AuNPs and *S. aureus* might be stronger than that between FITC – Lys and *S.*

aureus according the results shown in Fig. 2. If PEI – AuNPs bind to bacteria firstly, the steric hindrance of PEI – AuNPs also might hindered the recognition of Lys to *S. aureus*.

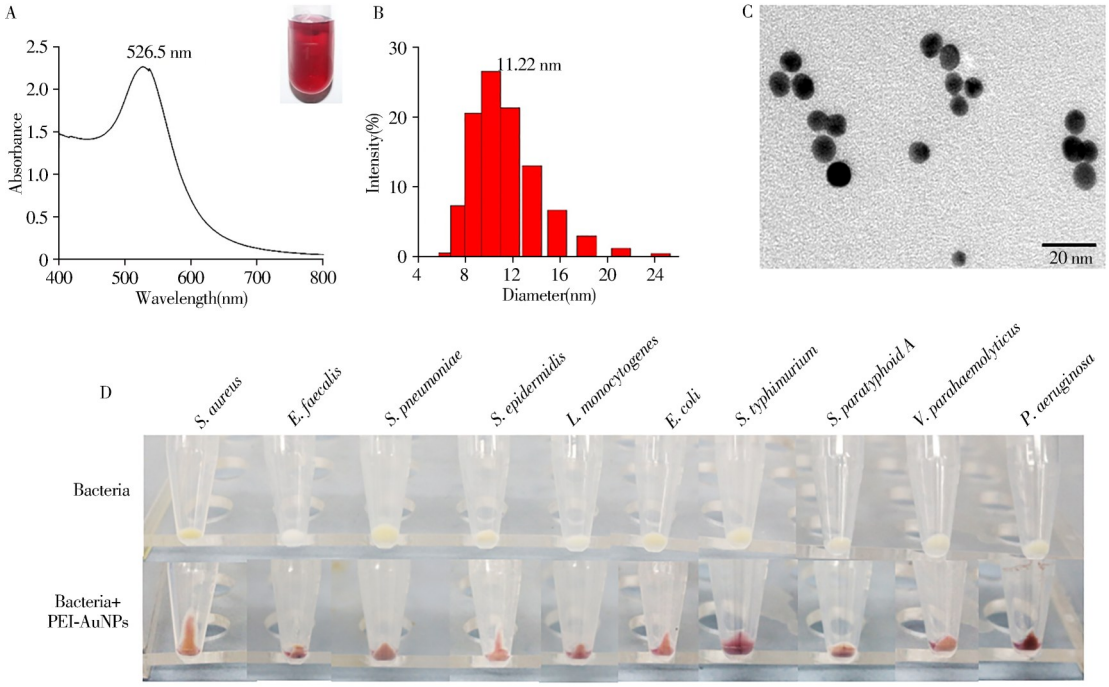

Fig. 3　UV – Vis absorption spectrum(A), hydrodynamic size distribution(B) and TEM image(C) of PEI – AuNPs; digital photos of each sample treated by centrifugation(D)
the inset in Fig. 3A is a digital photo of PEI – AuNPs

2.4　Feasibility of detecting *S. aureus* based on the FRET strategy

The feasibility of detecting *S. aureus* based on the proposed strategy was further verified by setting different control groups of blank(FITC – BSA protein and negative charged AuNPs were employes). Compared with the blank group(FITC – Lys + PEI – AuNPs), the fluorescence of the system (*S. aureus* + FITC – Lys + PEI – AuNPs) was quenched(Fig. 4), with ΔF of 1 877 ($\Delta F = F_0 - F$, where F_0 and F are the fluorescence intensities before and after adding *S. aureus*). However, other control groups shown no difference, suggesting that FRET strategy is feasible.

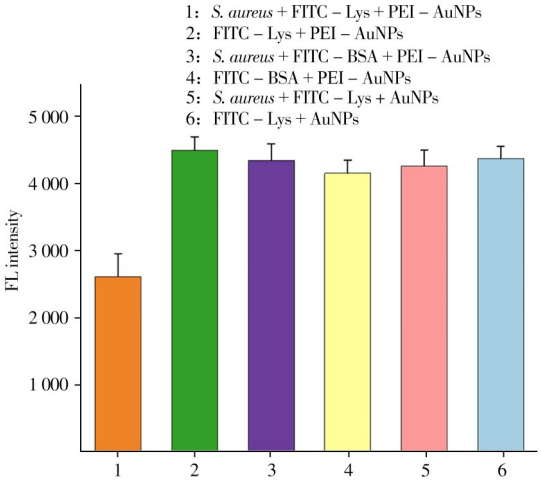

Fig. 4　Fluorescence intensity of different probes after incubating with or without *S. aureus*

2.5　Detection of broad spectrum bacteria

The proposed strategy for detecting broad spectrum bacteria was studied by employed both G^+(*S. pneumoniae*, *E. faecalis*, *S. epidermidis* and *L. monocytogenes*) and G^- bacteria (*E. coli*, *S. typhimurium*, *S. paratyphoid A*, *V. parahaemolyticus* and *P. aeruginosa*) as models. As shown in Fig. 5, ten different types of bacteria showed obvious fluorescence intensity after treated with FITC – Lys and PEI – AuNPs. More interestingly, the fluorescence intensity of the group containing *E. faecalis* showed the greatest change. The difference value of fluorescence signal is related to the content of peptidoglycan in cell wall, the amount of charge, the surface area of bacteria and hydrophobic interaction etc[59]. The cell wall of G^+ bacteria is mainly peptidoglycan, while the main component of G^- bacteria is lipopolysaccharide, with only two thin layers of peptidoglycan in the inner layer, so the content of peptidoglycan of G^+ bacteria is higher than that of G^- bacteria. FITC – Lys can specifically act on peptidoglycan

of bacteria, so FITC – Lys is easier to label G^+ bacteria, especially *E. faecalis* and *S. aureus*[60]. Because *E. faecalis* is G^+ bacteria and has more negative charges, so there are more fluorescence donors and receptors bound to *E. faecalis*, showing the strongest fluorescence signal.

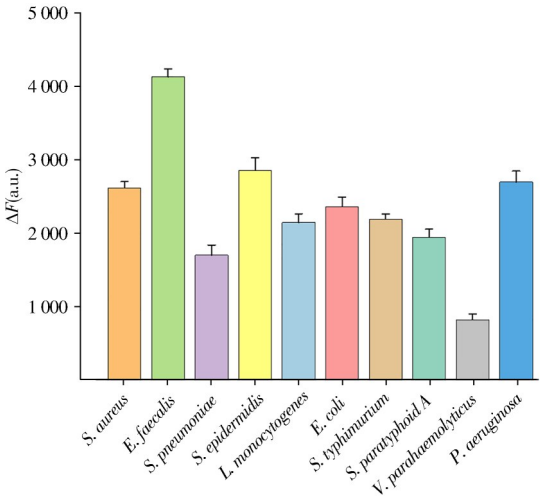

Fig. 5 Fluorescence intensity change(ΔF) of different types of bacteria treated with FITC – Lys and PEI – AuNPs

2.6 The interference experiment

To verify the application of the proposed sensor of broad spectrum bacteria in real samples, the potential interference of protein, metal ions, sugar and vitamins in real samples(taking milk and juice as examples) on the assay was investigated. According to the published work[61 – 62], the contents of main protein and ion in milk and the contents of sugar and vitamins in juice were as following: casein(28 g/L), β-lactoglobulin(3.5 g/L), bovine albumin(0.35 g/L), Ca^{2+}(1.04 g/L), PO_4^{3-}(0.73 g/L), K^+(1.09 g/L), Na^+(0.37 g/L), glucose(4.6 g/L), sucrose(9.8 g/L), and ascorbic acid(0.15 g/L). When the above-mentioned substances were added into buffer and subjected to analysis with PEI – AuNPs and FITC – Lys, we found some substances really could bring interference. Then all the substances were ten-times diluted and subjected to analysis again. Compared with the buffer sample and the group with *S. aureus*, all the tested substances showed no obvious interference, suggesting that the proposed method could be applied for detecting bacteria in real sample with appropriate dilution.

3 Conclusions

In summary, we have developed a universal platform for rapid and simple bacteria detection by using FRET sensing in which the donor of FITC – Lys and the receptor of positively charged AuNPs was employed. The proposed strategy showed feasibility of detection of broad spectrum bacteria.

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