Antibiotic Screening

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# Rapid, Simple, and High-Throughput Antimicrobial Susceptibility Testing and Antibiotics Screening**

*Chunlei Zhu, Qiong Yang,* Libing Liu, and Shu Wang**

The increasing number of pathogenic microorganisms with antibiotic resistance is a growing and increasingly serious global public health problem, especially as regards recent reports of the resistivity of *Enterobacteriaceae* conferred by New Delhi metallo-β-lactamase 1 (NDM-1).[1,2] Thus, antimicrobial susceptibility assessment and new antibiotics screening are of extreme importance.[3] Traditional methods include disk diffusion, agar dilution, antibiotic gradient disks, and—most commonly employed—broth microdilution.[4] The minimum inhibitory concentration (MIC) of a drug is then determined by visually judging liquid turbidity. Since growth-based susceptibility assays are all dependent on the growth of the microorganism, a time span of 24–48 h is required to reach the extent of visual resolution. Moreover, it is difficult to correctly and reproducibly decide the endpoints of growth.[5] To circumvent these deficiencies, several other strategies have been reported, including luciferase reporter phages,[6] electrochemical measurements,[7] real-time PCR,[8] and nanoparticle-based methods.[9] However, either the sophisticated instrumentation and operation steps or the unsatisfactory sensitivity and reproducibility limit their further application in an automated and high-throughput way.

Conjugated polymers (CPs) are characterized by a delocalized electronic structure, and the excitations can be efficiently transferred to lower energy electron- or energy-acceptor sites over long distances to super-quench the fluorescence of CPs or amplify the signal of acceptors.[10,11] In recent years, we and others have taken advantage of the excellent light-harvesting ability of CPs to enhance the sensitivity of biological detections.[10–20] Herein we demonstrate a simple, rapid, sensitive, accurate, and cost-effective system using CP–acceptor FRET pairs for evaluating antimicrobial susceptibility and screening antibiotics in a high-throughput fashion.

Scheme 1 shows the mechanism for antimicrobial susceptibility assessment and antibiotics screening. The electrostatic complex of cationic polyfluorene (PFP) and negatively charged fluorescein (FL; Figure 1a) is the sensing component. PFP, with maximum emission at 420 nm, exhibits excellent spectral overlap with fluorescein ($\lambda_{abs} = 488$ nm), thus leading to efficient fluorescence resonance energy transfer (FRET) between them upon selective excitation of PFP at 380 nm.[21–23] In the presence of ineffective antibiotics, the bacteria exhibit exponential growth. Because the surface of the bacteria is negatively charged,[24] the addition of bacteria culture into the PFP/FL pair will displace fluorescein from PFP by competitive electrostatic interactions, thus weakening FRET from PFP to fluorescein. In the presence of effective antibiotics, the proliferation of bacteria is suppressed and the PFP/FL pair is not affected by the bacterial culture, leaving the system with its original green fluorescence (characteristic color of FL). By triggering the change of emission color or FRET signal, it is possible to test antimicrobial susceptibility and screen new antibiotics.

Figure 1a shows the emission spectra change before and after addition of Gram-negative bacteria *E. coli* into the detection system. The addition of *E. coli* significantly enhances the PFP emission at 420 nm and decreases that of fluorescein at 525 nm. Apparent emission color transition from green to blue was observed (Figure 1b), indicating the visual detection. To show the system works with all types of microorganisms, Gram-positive bacteria *B. subtilis* and fungi *C. albicans* were also tested. Similar results were obtained (Figure 1c,d). Note that it is not possible to differentiate different types of bacteria.

The standard curve used for quantitative determination of *E. coli* amount was plotted using Equation (1), in which $RC_{FRET}$ represents the relative change of FRET ratio, and "$FRET_0$" and "FRET" are the initial and final FRET ratio of $I_{525nm}/I_{420nm}$, respectively (Figure 2b). Through such mathematical processing, both nonspecific external interferences and unparallel values from the initial FRET ratio can be eliminated at the same time, thus ensuring the accuracy and reproducibility of the method. The minimal detection limit of *E. coli* is $4 \times 10^4$ colony forming units (cfu), which is beneficial for the sensitivity of FRET-based assays.

$$RC_{FRET} = 1 - \frac{FRET}{FRET_0} \quad (1)$$

The antimicrobial susceptibility was tested and a qualitative plot was obtained (Figure 2b) under various concentrations of ampicillin. As the concentration of ampicillin increases from 2 to 80 μM, the growth of bacteria was gradually inhibited. The MIC and $IC_{50}$ can be simultaneously determined to be 8 μg mL$^{-1}$ and 3 μg mL$^{-1}$, respectively, which are consistent with previous reports, thus demonstrating the accuracy of our FRET-based method.[9] Note that it takes less than 4 h to obtain the identical result, greatly shortening the assay time.

[*] C. Zhu, Dr. Q. Yang, Dr. L. Liu, Prof. S. Wang
Beijing National Laboratory for Molecular Sciences
Key Laboratory of Organic Solids, Institute of Chemistry
Chinese Academy of Sciences, Beijing, 100190 (P. R. China)
E-mail: yangqiong@iccas.ac.cn
wangshu@iccas.ac.cn

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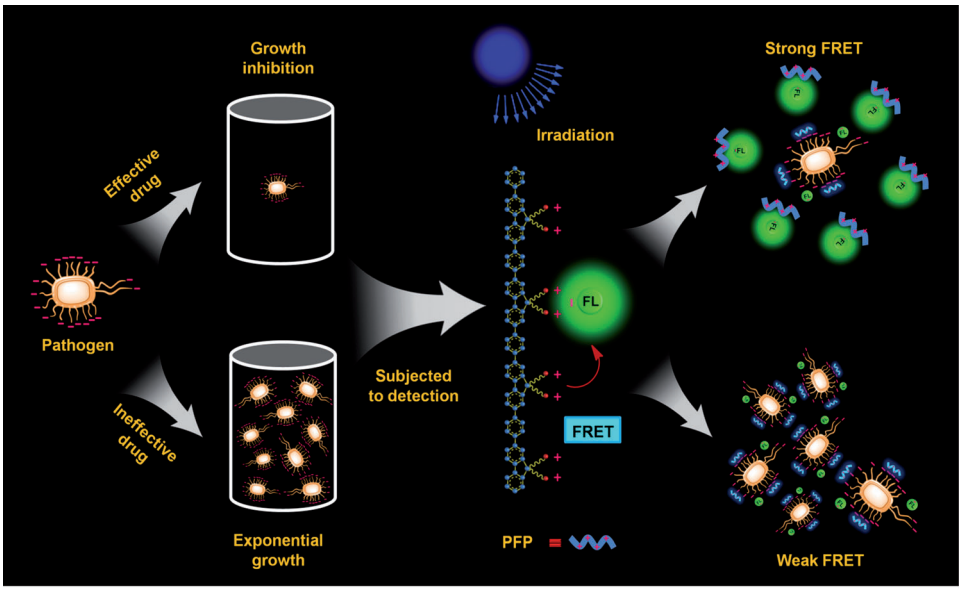


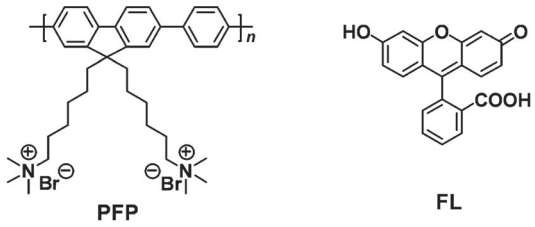


***Scheme 1.*** Schematic representation of antimicrobial susceptibility assessment and the chemical structures of cationic PFP and fluorescein (FL).

***Table 1:*** Results of susceptibility of Amp$^r$ *E. coli* towards various antibiotics.[a]

| Antibiotics | PEN | STM | AMP | CAR | TCN | GEN | CHL | ERM | CFX |
|---|---|---|---|---|---|---|---|---|---|
| $IC_{50}$ [μg mL$^{-1}$] | ≥128 | 64 | >128 | >128 | <1 | 6 | <1 | 4 | <1 |
| MIC [μg mL$^{-1}$] | >128 | >128 | >128 | >128 | <2 | 8 | <2 | 6 | 22 |
| Susceptibility | R | R | R | R | S | S | S | S | R |

[a] S: susceptible; R: resistant.

The susceptibility of a drug-resistant bacterium toward nine different antibiotics was investigated in a high-throughput format for large-scale drug screening. Herein, the ampicillin-resistant *E. coli* (Amp$^r$ *E. coli*) is used as model bacterium. To integrate with the automated microplate reader, a 96-well black microplate was used to favor high-throughput screening. Figure 3a shows a schematic illustration of fluorescence pattern generation on a microplate corresponding to a series of antibiotics with different efficacy. For effective drugs, the initial green fluorescence is maintained, while for ineffective drugs, the green fluorescence changes to blue. A standard curve for the microplate reader was also plotted after incubating PFP/FL pair and Amp$^r$ *E. coli* with different concentrations (Figure 3b). Subsequently, Amp$^r$ *E. coli* was grown in the presence of antibiotics with varying concentrations at 37 °C for 4 h, and then was subjected to fluorescence measurement to obtain the plots of bacteria amount versus drug concentration (Figure 3c). The corresponding susceptibility and values of MIC and $IC_{50}$ of Amp$^r$ *E. coli* toward various antibiotics are listed in Table 1. The nine drugs can be generally divided into three classes: super-effective (CHL, TCN), effective (CFX, ERM, GEN), and ineffective (AMP, CAR, PEN, STM). In other words, Amp$^r$ *E. coli* is susceptible to the all drugs in both the super-effective and effective groups and is resistant to the ones in the ineffective group. Since AMP, CAR, and PEN all belong to β-lactams, they are impotent to inhibit the growth of Amp$^r$ *E. coli* as a result of the expression of β-lactamases that are able to hydrolyze the β-lactam ring and mediate the drug resistance.[25] CFX is a third-generation cephalosporin which is relatively stable to β-lactamases; therefore, the better antimicrobial effect is attained. All other non-β-lactams exhibit low MIC and $IC_{50}$ values, with the exception of STM (no MIC interpretive standards are reported even for Clinical and Laboratory Standards Institute (CLSI)),[26] indicative of the high efficacy.

In conclusion, a rapid (within 4–5 h), sensitive, and quantitative new method for assessing antimicrobial susceptibility has been developed. The method is also robust enough to be used in high-throughput screening. No complicated instrumentation and expensive agents are utilized, making the method cost-effective. This new method is very helpful to defend the threats posed by pathogens and is beneficial for developing new drugs with brand-new antimicrobial mechanisms.

  

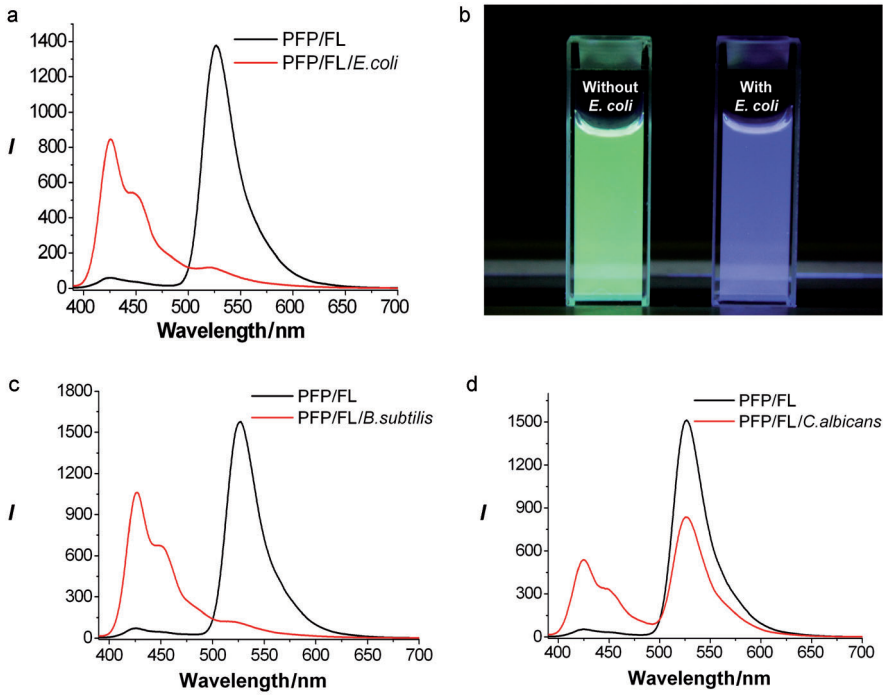


***Figure 1.*** a) Emission spectra of PFP/FL pair in the absence and presence of *E. coli*. b) A photograph of PFP/FL blend in the absence and presence of *E. coli* under UV light ($\lambda_{max}$ = 365 nm). c,d) Emission spectra of PFP/FL pair in the absence and presence of Gram-positive bacteria *B. subtilis* (c) and fungi *C. albicans* (d). The excitation wavelength is 380 nm. The measurements were performed in aqueous solution.

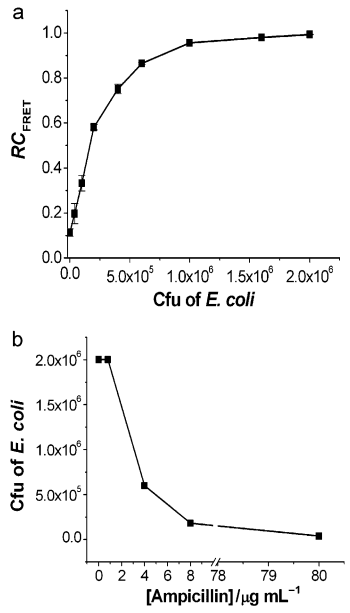


***Figure 2.*** a) A standard curve for quantitative determination of *E. coli* amount. b) Plot of *E. coli* amount versus the ampicillin concentration. The excitation wavelength is 380 nm. The measurements were performed in aqueous solution. Error bars represent the standard deviation of three experiments.

## *Experimental Section*

Materials and measurements: PFP was synthesized according to the procedure in the literature.[23] Fluorescein (FL) was purchased from Sinopharm Chemical Reagent Beijing Co., Ltd. Fluorimeter cuvettes were purchased from Sigma–Aldrich Inc. Penicillin G Na (PEN), streptomycin sulfate (STM), ampicillin Na (AMP), carbenicillin $Na_2$ (CAR), tetracycline HCl (TCN), gentamycin sulfate (GEN), chloramphenicol (CHL), erythromycin (ERM), and cefotaxime Na (CFX) were purchased from Xinjingke Biotechnology Co., Ltd (Beijing, China). 24-well cell culture plates and 96-well black microplates (for fluorescence assays) were purchased from Corning Inc. The liquid medium and double distilled water ($ddH_2O$) were autoclaved at 120 °C for 15 min. *Escherichia coli* (*E. coli*, TOP10) was purchased from Beijing Bio-Med Technology Development Co., Ltd. and transfected with ampicillin-resistant plasmids (pcDNA3, Invitrogen). *Bacillus subtilis* (*B.subtilis*) and *Candida albicans* ATCC 10231-(CA10231) were obtained from China General Microbiological Culture Collection Center. Fluorescence spectra were measured on a Hitachi F-4500 fluorometer equipped with a Xenon lamp excitation source. Photographs of the polymer solutions were taken using a Canon EOS 550D Digital camera under a hand-held UV lamp (ZF-7A, Shanghai Gucun Electron Optic Instrument Factory) with $\lambda_{max}$ = 365 nm. For high-throughput drug screening, the fluorescence signals were read on a microplate reader (BIO-TEK Synergy HT, USA) equipped with an excitation filter of 380/20 nm. The emission filters were 440/30 nm for PFP and 528/20 nm for FL.

Preparation of bacterial solutions: A single colony of *E. coli* (Gram-negative) on a solid Luria broth (LB) agar plate was transferred to 5 mL of liquid LB culture medium and grown at 37 °C overnight. Bacteria were harvested by centrifuging (4000 rpm for 5 min) and washing with $ddH_2O$ for three times. The supernatant was discarded and the remaining *E. coli* was resuspended in $ddH_2O$ and diluted to an optical density of 1.0 at 600 nm ($OD_{600}$ = 1.0). For ampicillin-resistant *Escherichia coli* ($Amp^r$ *E. coli*), liquid LB culture medium was supplemented with 100 μg $mL^{-1}$ ampicillin. For *B. subtilis* (Gram-positive), the culture medium was replaced by beef-extract peptone yeast-extract (BPY). For *C. albicans* (fungi), the culture medium was replaced by yeast-extract peptone dextrose (YPD), and the incubation temperature was 25 °C.

Spectral measurements for microorganism-mediated breakage of fluorescence resonance energy transfer (FRET): 1 mM PFP and 1 mM FL were diluted 10-fold and 60-fold, respectively, with $ddH_2O$. To 1 mL of $ddH_2O$ in a fluorimeter cuvette was serially added 4 μL of dilute solution of PFP and FL. Then the spectrum was measured. Next, a solution (20 μL) of pathogens (*E. coli*, *B. subtilis*, and *C. albicans*) with different colony-forming units (cfu) was added to the above system. After incubation at 37 °C for 17 min, the fluorescence spectrum was measured again. Fluorescence intensity changes at 420 and 525 nm were recorded to calculate the FRET ratio. The excitation wavelength was 380 nm.

Artificial contamination of LB medium for drug screening: fresh LB medium was added to a 24-well plate, and then drugs to be screened were introduced to the medium and serially diluted two-

  

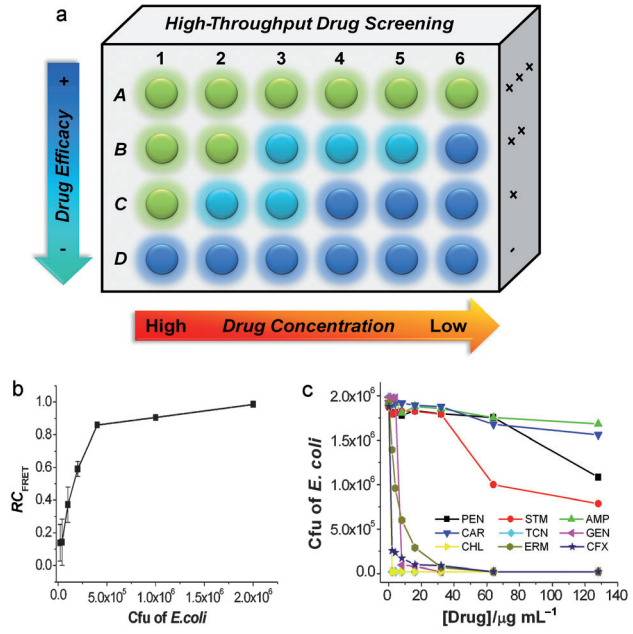


***Figure 3.*** a) Schematic representation of fluorescence pattern on a microplate corresponding to various antibiotics with different efficacy. In the diagram, 1–6 represent the gradually decreased drug concentration, and A–D represent different antibiotics with decreasing drug efficacy. Green, light blue, and dark blue wells indicate complete, partial, and scarce inhibition. b) Standard curve measured on a microplate reader with respect to the model Amp$^r$ *E. coli* of various concentrations. c) Plot of Amp$^r$ *E. coli* amount versus antibiotics concentrations. The excitation wavelength is 380 nm. Error bars represent the standard deviation of three experiments. See the Experimental Section for definitions of drug abbreviations.

fold. The final volume of each well was 500 μL. Next, medium containing drugs with different concentrations was inoculated with pathogens (*E. coli* and Amp$^r$ *E. coli*, 8 μL $OD_{600} = 0.5$) to a final concentration at about $2–6 \times 10^5$ cfu mL$^{-1}$. The artificially inoculated samples were cultured at 37 °C for 3–4 h. Fresh LB medium (500 μL) was used as the blank control. After 3–4 h of incubation, 200 μL of culture was taken out and centrifuged at 6900 *g* for 1 min. Then the obtained pellets were washed by $ddH_2O$ once and suspended in $ddH_2O$ (200 μL) to be analyzed.

High-throughput drug screening: 1 mM PFP and 1 mM FL were diluted 10-fold and 60-fold, respectively, with $ddH_2O$. Dilute solution of PFP and FL (90 μL) was serially added to 5% ethanol in $ddH_2O$ (6 mL). The addition of 5% ethanol is to stabilize the fluorescence signal in 96-well plates. Suspensions obtained as described above (20 μL) were added to three parallel wells of a 96-well plate, and then test solution (PFP/FL blend, 200 μL) was introduced to each well containing bacterial suspensions. After incubation at room temperature (RT) for 10 min, the fluorescence values corresponding to the emission of PFP and FL were read on a microplate reader to calculate the FRET ratio.

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