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Anal. Chem., **Just Accepted Manuscript** • Publication Date (Web): 22 May 2019

Colorimetric and electrochemical detection of Escherichia coli and their antibiotic resistance based on p-benzoquinone-mediated bioassay

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ABSTRACT: The facile and economical identification of pathogenic bacteria, especially their antibiotic-resistance, is crucial in the realm of human health and safety. The presence of Escherichia coli (E. coli) is considered as an indicator of water contamination and is closely related to human health. Herein, inspired by the biocatalysis of bacterial surface, we developed a simple and cost effective colorimetric- and electrochemical-based bioassay, which is capable of analyzing both the presence of E. coli and its relative level of antibiotic resistance. In this approach, p-benzoquinone is used as a redox mediator to monitor the bacterial concentration and specifically distinguish E. coli from four other common clinical bacteria, namely Staphylococcus aureus (S. aureus), Enterococcus faecalis (E. faecalis), Salmonella pullorum (S. pullorum), and Streptococcus mutans (S. mutans). Visible color change, captured with a smartphone using a "light box", without relying on any complex instruments, can reflect the concentration of bacteria. The accurate quantification of E. coli was investigated with electrochemical system in the concentration ranges of 1.0×10^3 to 1.0×10^9 CFU/mL. We further demonstrated the capability of the presented biosensor in identifying drug-resistant bacteria with two artificially induced antibiotic-resistant bacteria. Therefore, the presented bioassay is not only capable of detecting E. coli with high sensitivity and specificity, but also provides a rapid solution to evaluate E. coli antibiotic-resistance.

Bacteria contamination and its rapidly evolving resistance to antibiotic are major public health threats and substantial financial burdens on healthcare, causing more than 10 million deaths worldwide each year [1,2]. Therefore, the sensitive detection and identification of bacteria concentration and antibiotic resistance is a significant focus in environmental science, food safety, clinical diagnosis, and treatment. Obtaining results with traditional bacterial detection methods, including bacteria cell separation, culturing, and counting [3], often require a minimum of two weeks and is longer when ascertaining the sample's resistance to specific antibiotics. Enormous efforts have been made towards developing enabling technologies to meet the growing demand for rapid and sensitive detection of bacteria. Commonly used methods in laboratories include polymerase chain reaction (PCR) [4], surface plasmon resonance (SPR) [5], enzyme-linked immunosorbent assay (ELISA) [6], surface-enhanced Raman scattering (SERS) [7], mass spectrometry [8], microarrays, and biosensors [9]. Although these methods are accurate and sensitive, they are expensive, time-consuming, labor-intensive, and require professional instruments, resulting in poor adaptability for field analysis and presenting the need to develop innovative, inexpensive, rapid, and simple methods for the detection of bacteria.

Recently, colorimetric- and electrochemical-based platforms are receiving widespread attention due to their remarkable ability at detecting pathogens in different matrices [10]. Colorimetric based detection provides simple and visual analysis without complex operation or instruments. Many simple, sensitive, and cost-effective colorimetric methods based on various nanomaterials or enzyme catalysis reactions are employed for bacterial detection to prevent threatening diseases [11 12]. Such as a D-amino acid-capped gold nanoparticle biosensor for colorimetric detection of bacteria, where color change was adopted to distinguish between Staphylococcus aureus (S. aureus) and methicillin-resistant S. aureus (MRSA) [13]. In addition to colorimetric-based detection, electrochemistry provides lower bacteria detection limits, with the desired ability to quantify, and not constrained by sample background color [14]. There are extensive reports on simple, rapid, and sensitive detection of bacteria based on several different principles relying on electrochemical techniques [15]. For instance, a fully automated microfluidic-based electrochemical sensor for real-time bacteria detection was recently developed [16]. It utilizes HRP-TMB interaction and nanomaterial amplified immunoassays to achieve quantification of pathogen in the concentration range from 0.99×10^4 to 3.98×10^9 colony forming units/mL (CFU/mL). These methods enable sensitive and selective detection of bacteria but require expensive modification processes with temperature-sensitive enzymes, nanomaterial synthesis, and lengthy analysis procedures, which all limit practical utilization.

P-benzoquinone (BQ) is well-known as a lipophilic redox mediator, which can be used in electrochemical biosensor for determining toxicity, glucose, and substrate-oxidizing activity [15,17]. The use of BQ-mediated recognition elements in biosensor systems has become mature and common. However, there is no existing literature that adopts BQ for Escherichia coli (E. coli) quantity detection and antibiotic resistance analysis. The

presence of E. coli is considered as an indicator of water contamination, and high concentrations of E. coli can lead to serious illnesses, such as diarrhea, urinary tract infections, meningitis, anemia, and kidney failure [18]. E. coli concentrations, as low as 10^4 CFU/mL, can be detect by microspheres coated with overoxidized polypyrrole via microscopic images [19]. Here, we report a new BQ-mediated based E. coli detection system, capable of both colorimetric detection with visual analysis and electrochemical detection with easy quantification. Our colorimetric and electrochemical analytic detection assay can specifically identify E. coli from four other types of Gram-positive (S. aureus, E. faecalis, S. mutans) and Gram-negative (S. pullorum) bacteria. This method realizes the quantification of E. coli in the concentration ranges from 1.0×10^3 to 1.0×10^9 CFU/mL. Compared to available electrochemical-based bacterial biosensors, our method is easier to operate without introducing any additional sample processing steps. It is an enzyme-free strategy and precludes the interference of enzyme-based instability and complex nanomaterial synthesis. In addition, visible color change, without relying on any complex instruments, reflecting bacteria concentration can be observed by the naked eye. The feasibility of the proposed bioassay in determining antibiotic-resistant bacteria is examined and further demonstrated using two artificially induced antibiotic resistant bacteria (Trimethoprim resistant E. coli and Erythromycin resistant E. coli). The assay introduced here can be used as a rapid and cost-effective method to specifically identify and quantify E. coli while also distinguish its antibiotic resistance.

Methods and materials

Materials and apparatus. BQ, Trimethoprim, and Erythromycin were acquired from Sangon Biotech (China), and the phosphate buffered saline (PBS, 10 mM, pH 7.4) was acquired from Thermo Fisher Scientific (USA). The Luria Bertani broth (LB, 10% Peptone, 5% yeast extracts, and 10% sodium chloride) was obtained from Sinopharm Chemical Reagent (China) and was adjusted to the desired pH (7.4) with 6 M NaOH (Sangon Biotech, China), then it was sterilized in a high-pressure steam camber at 121 ℃ for 35 min. Electrochemical measurements were performed with a CHI 660E electrochemical workstation (CH Instruments, USA) and a conventional three-electrode system consisting of an ITO/PET working electrode, a platinum counter electrode, and an Ag/AgCl (3 M KCl) reference electrode (Chenhua, China). The scan rate used for CV measurements was 0.1 V/s.

Microbial culture. E. coli (ATCC25922), E. coli O157:H7 (NCTC12900), E. faecalis (ATCC33186), S. mutans (UA159), S. aureus (ATCC29213), and S. pullorum (CVCC578) were used in the experiments. All bacteria were sustained in the LB medium. Bacterial cultures were grown overnight (12 h, 37 °C, shaking at 150 rpm). Then bacteria were harvested via centrifugation (Eppendorf 5804R) at 3600 rpm for 10 min and washed with PBS three times. The remaining bacterial pellets were suspended in PBS for further use.

Antibiotic resistant bacteria induction. Induction experiments were conducted in Erlenmeyer flask with final volume of 100 mL. Wild type (WT) E. coli was inoculated into a series of Erlenmeyer flasks with a twofold antibiotic concentration gradient. The initial concentration was 50% minimal inhibitory concentration (MIC). The flasks were then placed into a shaker bath (150 rpm) at 37 ℃. After 24 h, OD_{600} values of each flask were measured and the strain with the highest antibiotic concentration permitting bacterial growth was propagated into another round of antibiotic resistance incubation with higher antibiotic concentration gradient. After 5 rounds of selection, E. coli was collected and marked as low-antibiotic resistant, and after a total of 10 rounds of selection, E. coli was collected again and marked as high-antibiotic resistant.

BQ optimization. Different concentrations of BQ (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mM) were prepared with PBS (pH 7.4). Then we diluted the E. coli with PBS to 1.0×10^9 CFU/mL. The assays were performed in 96-well microplates for colorimetric detection and centrifuge tubes for electrochemical detection. 100 µL bacteria (1.0×10^9 CFU/mL) and 100 µL BQ at various concentrations (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mM) were added into the plates. After reacting for 1 h, the color change was recorded. In the meantime, 3 mL bacteria (1.0×10^9 CFU/mL) and 3 mL BQ at various concentrations were prepared and incubated in centrifuge tubes for 1 h. Then, the samples were centrifuged at 3600 rpm for 10 min. Supernatants of these samples were used for electrochemical detection.

Bacterial detection. Bacterial strains, including E. coli, E. coli O157:H7, E. faecalis, S. mutans, S. aureus, S. pullorum, and two strains of antibiotic resistant bacteria (Trimethoprim resistant E. coli and Erythromycin resistant E. coli), were diluted to desired concentration with PBS. Then, we added 100 µL of BQ (optimal concentration) and 100 µL of various bacterial strains with different concentrations into the 96-well microplates for colorimetric detection. After 1 h of reaction, the color change in the 96-well plate was recorded. Samples for electrochemical detection were prepared by mixing 3 mL BQ (6 mM) solution and 3 mL bacteria suspense on. After incubating for 1h, the biocatalytic reaction was terminated by centrifugation at 3600 rpm for 10 min, and the sample supernatants were electrochemically analyzed.

Results and discussion

Feasibility of p-benzoquinone-mediated bioassay at detecting E. coli. Whole microbial cells and isolated enzymes are often used to construct biosensors and for redox compound conversions in bioreactors. In whole-cell biosensors, these compounds transfer electrons between microorganisms and electrodes, which can be used to monitor photo-synthetic activity or cell respiration [20]. The redox compounds, as electron mediators, can replace oxygen in the respiration enzymatic reaction and act as final electron acceptors for microbial cells [21]. The enzymatic reaction of cell respiration causes intact bacterial cells to behave as oxidoreductases, significantly reducing redox compounds, such as BQ, ferricyanide, dichlorophenolindophenol (DCIP), and other organic dyes [22]. During E. coli cell respiration, glucose reacts with an intact redox enzyme (DHase, such as D-glucose dehydrogenase). The enzyme is reduced by catalytic reactions, and the reductase supplies electrons to the electron acceptor (here BQ) directly or through an appropriate site on the respiratory chain. When the E. coli cells are appropriately poised as electrodes, BQ, as an electron mediator, will reduce to hydroquinone (HQ), which can be used to monitor the catalytic events (equation below). The catalyzed reaction with E. coli cells follows the present cyclic voltammetry method [15].

$$\text{P - benzoquinone} + e^- \rightarrow \text{Hydroquinone}$$

In this assay, the enzymatic events (cell respiration) of E. coli reduces BQ to HQ, which with remaining BQ to produce a red complex: quinhydrone. At suitable BQ concentrations, the color change can indicate the concentration of E. coli. In addition, remaining BQ is reduced on the surface of the working electrode, resulting in a current flow. A conventional three-electrode system can be used to monitor the concentration of BQ for quantitative analysis, which can indicate the concentration of E. coli. Figure 1 illustrates a schematic model of the catalytic reaction by a bacterial cell.

Optimized concentrations of BQ. To evaluate the effect of BQ concentration on the sensitivity of the proposed assay, samples including various concentration of BQ (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mM) and E. coli (1.0×10^9 CFU/mL) were tested. Figure 2A shows the color change after 1 h of incubation, as shown, the red color was deeper with higher concentrations of BQ. Figure 2B displays the obtained cycle voltammetry (CV) curves. The redox peaks of these curves were enhanced with increased concentrations of BQ. As control, CV curves of BQ at different concentrations mixed with the same volume of PBS are shown in Figure 2C. The redox peak shows a clear rise following the increased concentrations of BQ. To evaluate the difference between BQ with PBS and samples containing BQ and E. coli, oxidation peak differences are shown in Figure 2D. These values show a significant increase between 1 mM to 6 mM of BQ. After 6 mM, these values gradually decreased. Therefore, 6 mM was chosen as the optimal BQ detection concentration for E. coli due to its wide detection range.

Colorimetric and electrochemical detection of E. coli. Once the optimal BQ concentration was determined, E. coli was diluted to various concentrations (1×10^9, 1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4, and 1×10^3 CFU/mL) with PBS for further analysis. As shown in Figure 3A, obvious color gradient could be seen at different E. coli concentrations, from 1.0×10^4 through 1.0×10^9 CFU/mL. The color response could be observed by the naked eye or captured by a smartphone for accurate determination of bacteria concentration through RGB analysis (Figure S1). All photos were obtained via smartphone in a "light box" and processed with Python© to calculate the RGB values (Figure S2). The color in the 96-well plates were deeper with the increase of E. coli concentrations. The reaction with bacteria reduced the redox mediator BQ to HQ via the enzymatic event within the cells. The reducer mediator, HQ, then diffuses from the cell and could re-oxidize at an electrode poised at a sufficient oxidizing potential. The oxidation reaction was therefore selected for further study. To quantify the colorimetric response of E. coli, we used a conventional three-electrode system to monitor the samples. Figure 3B depicts the CV curves of the interfaces by plotting current signal vs. potential. The redox peak exhibited liner decrement with the increase of E. coli concentration from 1.0×10^3 to 1.0×10^9 CFU/mL. Their relationship function is represented as $I_{E.coli}$ (μA) = 0.00102 - 1.35297E-4 × $C_{E.coli}$ (μM) with R^2 of 0.99514 (Figure 3C). This method allows for the detection of E. coli down to 1.0×10^4 CFU/mL via simple visual readout or 1.0×10^3 CFU/mL via electrochemical detection.

To validate the performance of the presented assay in real world water samples, we tested unfiltered tap water. After three washes, E. coli pellets were mixed with the tap water sample to ensure that the inoculated water contained 1.0×10^8 CFU/mL concentration of E. coli. Figure S3 shows the color response and CV curves of the water sample. The control group contained the same concentration of E. coli diluted in PBS. As seen in Figure S3, there is negligible difference both visually and graphically, validating the applicability of our assay to test real word water sample.

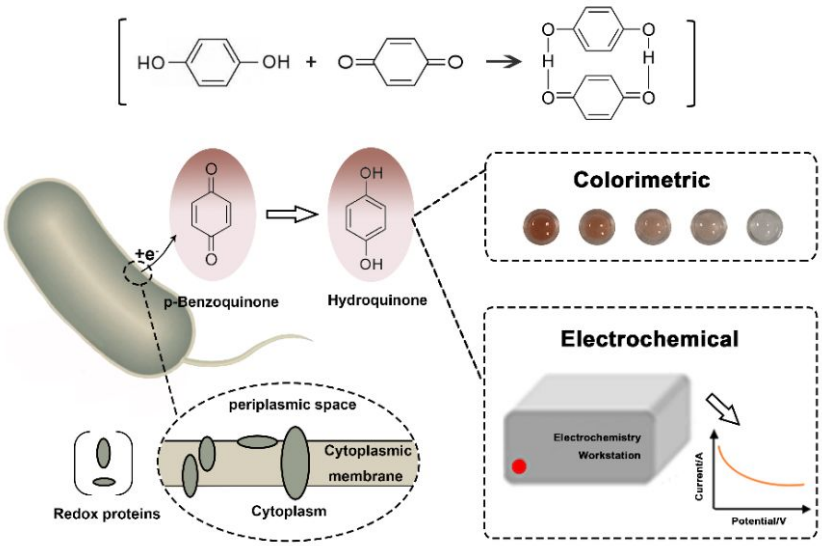

Figure 1. Schematic illustration of E. coli detection using BQ-mediated colorimetric and electrochemical assay. Due to the enzymatic reaction of E. coli, BQ can be used as an electron acceptor to receive electrons from a bacterial respiratory chain. The reduction product HQ reacts with remaining BQ to produce a red complex: quinhydrone. Remaining BQ is reduced on the surface of the working electrode, resulting in an electric current. The colorimetric and electrochemical signals are used to quantify E. coli.

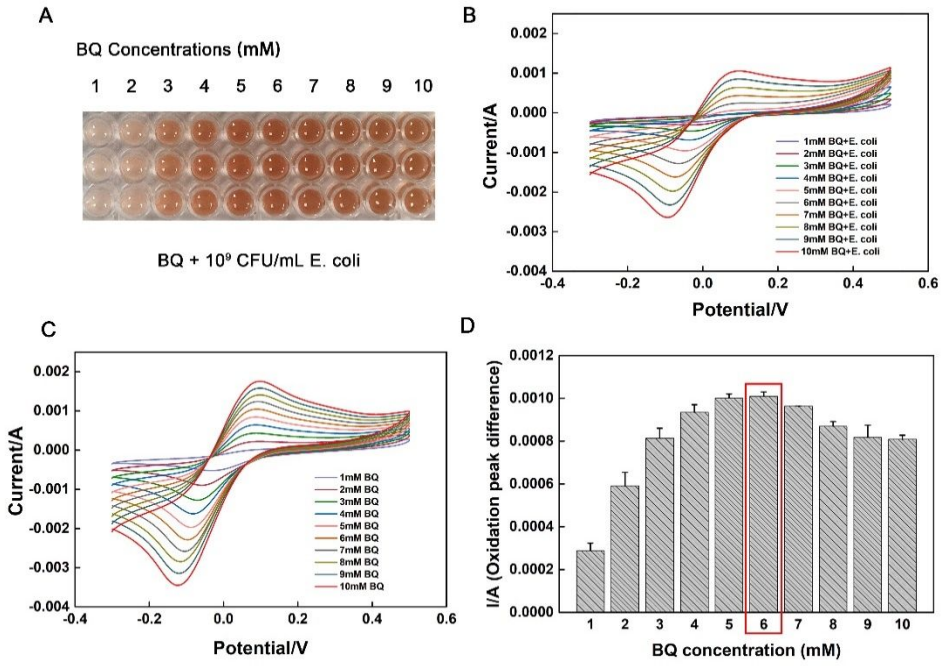

Figure 2. Optimization results of BQ concentration. (A) Colorimetric optimization results of BQ after incubating with E. coli. (B) CV curves of BQ at different concentrations after incubating with E. coli. (C) CV curves of BQ at different concentrations after incubating with PBS. (D) Comparison result of BQ after incubating with E. coli and PBS, 6 mM was chosen as the optimal detection concentration for E. coli. The mean and standard deviation of all concentrations are from three replicated experiments.

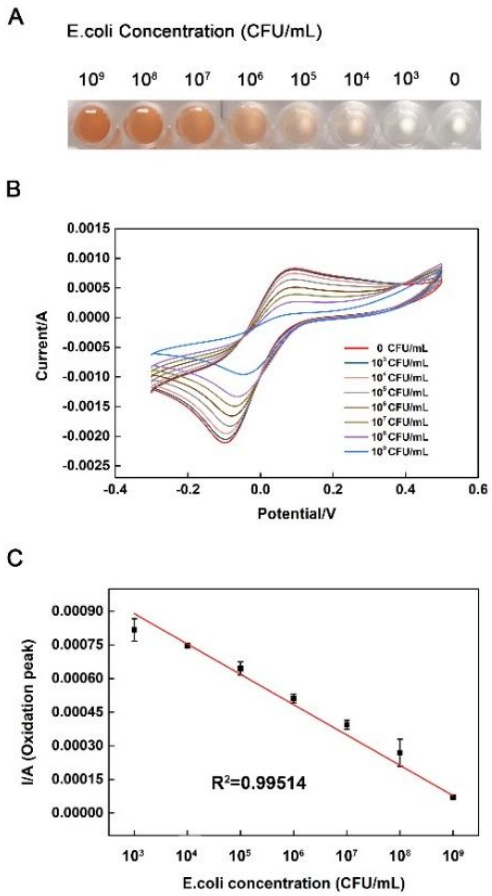

Figure 3. Colorimetric (A) and electrochemical (B) detection of E. coli in the concentration range of 1.0×10^3 – 1.0×10^9 CFU/mL. (C) Calibration curve shows detection of E. coli. The mean and standard deviation of all concentrations are from three replicated experiments.

Assay selectivity. To evaluate the selectivity of this assay, five types of bacteria, namely E. coli, E. faecalis, S. mutans, S. aureus, and S. pullorum, were tested at different concentrations (1.0×10^9, 1.0×10^8, 1.0×10^7, and 1.0×10^6 CFU/mL). Figure 4A shows the colorimetric response of BQ (6 mM) by incubating with various types of bacteria. These Gram-positive (S. aureus, E. faecalis, S. mutans) and Gram-negative (E. coli, S. pullorum) bacteria are common in clinical samples. After 1 h of incubation, the color of samples that contained E. coli changed to red, whereas the color of other bacteria (E. faecalis, S. aureus, S. pullorum, and S. mutans) were unchanged. Figure 4B shows CV responses of a conventional three-electrode system after incubation with different types of bacteria at a concentration of 1.0×10^9 CFU/mL. The redox peak of E. coli decreased significantly, while the redox peaks of other bacteria did not change (Figure S4). Our data indicates that the presented approach can identify E. coli from other common clinical bacteria both visually and electrochemically.

In addition, E. coli O157:H7 is considered as the most important E. coli strain in food safety and public health [23]. We validated the performance of the presented method with this bacterial strain. Various concentrations (1×10^9, 1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4, and 1×10^3 CFU/mL) of E. coli O157:H7 were tested. As shown in Figure S5, an observable change in color and redox peak could be seen at different concentrations of E.coli O157:H7. This method allows for the

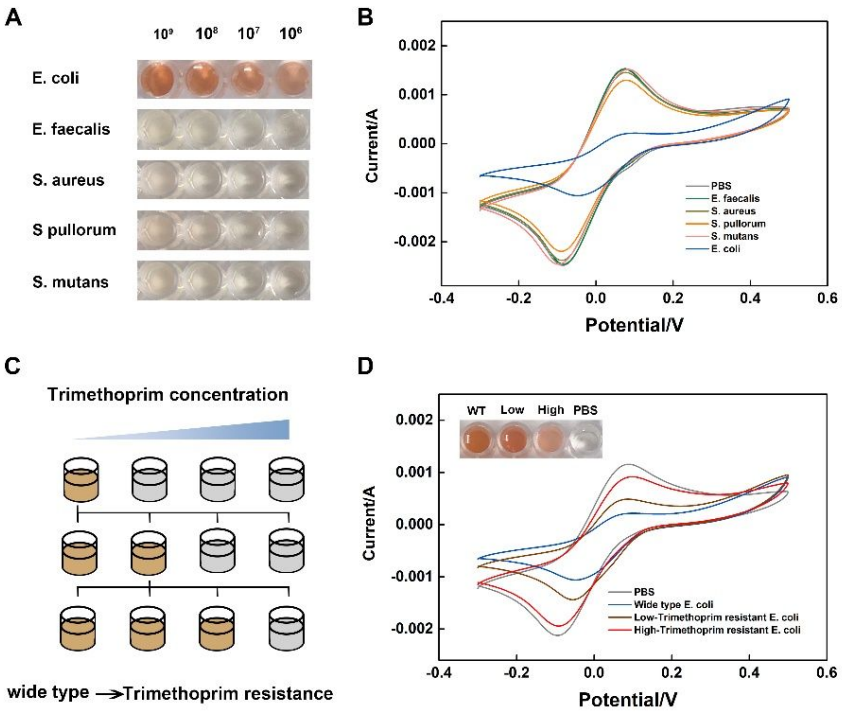

Figure 4. Colorimetric (A) and electrometrical (B) response of 6 mM BQ to different types of bacteria (E. coli, E. faecalis, S. mutans, S. aureus, and S. pullorum). After 1 h of incubation, only E. coli was specifically identified. (C) The process of Trimethoprim resistant bacterial induction. Two types of antibiotic-resistant bacteria (low-Trimethoprim resistant E. coli and high-Trimethoprim resistant E. coli) were artificially induced by continuous antibiotic stimulation. (D) Colorimetric response and CV curves of E. coli with varying levels of resistance to antibiotic.

detection of E. coli O157:H7 down to a concentration of 1.0×10^4 CFU/mL. Therefore, this assay could be used to detect different E. coli strains. However, as the redox mechanism is universal for different strands of E. coli, this assay is unable to distinguish between different strains.

Detection of antibiotic resistant bacteria. In addition to rapid and ultrasensitive detection of bacteria, identification of their relevant drug resistance is important in clinical applications. As discussed above, our method is capable of specific detection of E. coli. To further explore the effects of antibiotic-resistant bacteria on our method, we tested two other antibiotic-resistant bacteria (low-Trimethoprim resistant E. coli and high-Trimethoprim resistant E. coli) and WT E. coli (Figure S6). The resistant bacteria used in this study was artificially induced [24] (Figure 4C). Daily growth curves of these two resistant E. coli and WT E. coli were measured every 6 hours until the concentration of E. coli was stable. Figure S7 shows the growth curves for these three strains. MIC of these three bacteria were tested and shown in Figure S8. MIC of WT E. coli was 12.5 μg/mL, and the MIC of the other two artificially induced strains (low-Trimethoprim resistant E. coli and high-Trimethoprim resistant E. coli) were 120 μg/mL and 250 μg/mL, about 10- and 20-fold of resistance to Trimethoprim compare to its WT.

After successfully inducing E. coli with varying Trimethoprim resistance, we evaluated the proposed method for detection of antibiotic-resistance. To eliminate the influence of concentration levels on detection and reduce experimental variables, we tested these three strains at the same concentration (1.0×10^9 CFU/mL). After reacting with BQ for 1 h, the color of WT E. coli turned red, whereas, the color of antibiotic-resistant bacteria was shallower compared to that of WT bacteria. As the resistance of E. coli increased, the red became even shallower after reaction (Figure 4D). The redox peak shows a clear rise following the increased resistance in E. coli (Figure S9). This result may arise from the differences of some central function of cellular metabolism, which influences the substrate oxidizing activity of viable cells. When Trimethoprim acts on bacteria, it can inhibit the reduction of dihydrofolic acid to tetrahydrofolic acid by binding to dihydrofolate reductase, which inhibits bacterial DNA biosynthesis [25]. In inducing Trimethoprim resistance changed the substrate oxidizing activity of the bacteria.

To validate the effects of other forms of antibiotic resistant E. coli, we further induced E. coli with varying Erythromycin resistance with the same method above. Then we tested these three strains (low-Erythromycin resistant E. coli, high-Erythromycin resistant E. coli, and WT E. coli) at the same concentration (1.0×10^9 CFU/mL). Figure S10 and S11 show the CV curves and statistical analysis results of these three bacteria strains. The redox peak shows a clear upward trend following the increased Erythromycin resistance in E. coli. This result indicated that the effect of Trimethoprim was not unique in affecting the assay. Other forms of antibiotic resistance show a similar effect. This assay can potentially distinguish WT E. coli and various antibiotic resistant E. coli. In addition to the effects of antibiotics, we know from the literature that high concentrations of heavy metal ions, such as Cu2+, Ag+, Hg2+,

and Co2+, may affect the oxidizing activity of the bacteria [26]. Therefore, we recommend using our method for detect bacteria in non-heavy metal contaminated samples.

Conclusion

In summary, by utilizing the redox of BQ, this work reports a colorimetric and electrochemical based bioassay for specific, sensitive, and economical detection of the waterborne pathogen E. coli. This bioassay can profile E. coli antibiotic resistance and distinguish WT E. coli from antibiotic resistant E. coli with the same concentration. In this assay, electrochemical method is more sensitive, detecting E. coli concentrations as low as 1.0×10^3 CFU/mL within an hour, and outputs accurate quantitative results. For the colorimetric method, 1.0×10^4 CFU/mL concentration E. coli could be detected through an observable change in color, which can be captured via smartphone in a "light box". Though it is not as sensitive as the electrochemical method, the color response is easily observable by the naked eye or photos captured by a smartphone for accurate determination through RGB analysis in real time without any large-scale instruments. The advantages of using both methods (colorimetric and electrochemistry) together can overcome the disadvantages in either techniques, making the combined approach more applicable to a wider range of scenarios. Future developments of this method include reducing detection limits, increasing the selectivity of resistant bacteria, integrating this analytical method into paper-based devices, detection of drug resistance in real and complex samples, and finding other redox mediators (ferrocyanide, dichlorophenolindophenol, etc.) to identify different bacteria. We believe that this method provides a robust potential for simple and specific detection of E. coli and antibiotic-resistant E. coli.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website.

- The calibration curves of RGB values for varying E. coli concentrations. (Figure. S1)
- The diagram of the photo capture setup. (Figure. S2)
- Test results of contaminated tap water sample. (Figure. S3)
- Statistical analysis result of different types of bacteria. (Figure. S4)
- Colorimetric and electrochemical detection of E. coli O157:H7. (Figure. S5)
- Photo of WT E. coli and two kind of antibiotic-resistant bacteria. (Figure. S6)
- Growth curve for E. coli. (Figure. S7)
- Minimal inhibitory concentration (MIC) of E. coli. (Figure. S8)
- Statistical analysis results of different Trimethoprim-resistant E. coli. (Figure. S9)
- Colorimetric response and CV curves of different Erythromycin resistant E. coli. (Figure. S10)
- Statistical analysis result of different Erythromycin-resistant E. coli. (Figure. S11)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was supported by Science and Technology Innovation Zone (17-163-15-XJ-002-002-09), National Natural Science Foundation of China (31771088), Shanghai Municipal Science and Technology Project (17DZ2203400), and State Key Research and Development Plan (2017YFC0107603 and 2017ZX10203205-006-002).

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