A rapid detection system design for \textit{Escherichia coli} in food based on a nanoprobe and graphite electrode coupled with ATP bioluminescence technology

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\textbf{ABSTRACT} Food safety concerns owing to bacterial contamination have drawn significant attention; thus, the development of techniques for rapid and accurate detection of pathogens is an active research area. The development of a biological detection methodology based on techniques such as nanoprobe, graphene transparent electrode (GTE), or adenosine triphosphate (ATP) luminescence is described in this manuscript. Two system parts were designed; one can pump reagents precisely controlled by a microprogrammed control unit (MCU), whereas the other can rapidly process signals from a photomultiplier tube (PMT) by the hard module. Thus, the system works automatically and detects bacteria in food rapidly. The system not only uses the probe to capture and enrich \textit{Escherichia coli} via an antibody-antigen reaction but also enriches ATP using an electric field generated by the GTE to further improve the accuracy of the system. Compared with the other conventional approaches, this system can produce a linear correlation coefficient of up to 0.972 and meet the design demand. Moreover, detection can be completed within 20 min. The detectable bacterial concentration range is $10^2$–$10^6$ CFU/mL. Additionally, a series of curves obtained by measurements of different polluted food products such as drinks, meat, and grains show that the system has a satisfactory performance. It meets the requirements of a rapid, on-site detection system.

\textbf{INDEX TERMS} Food technology, fluorescence, rapid detection, optical signal detection

\textbf{I. INTRODUCTION} Human activities have resulted in urban pollution and industrial and agricultural contamination with pathogenic bacteria such as \textit{Escherichia coli} and \textit{Bacillus} species. Bacterial infections account for approximately 40% of all diseases \cite{1}, and food safety concerns from bacteria have become increasingly serious. Therefore, rapid and accurate detection of bacteria in food has become an urgent topic in the field of food safety research.

\textit{E.coli} is routinely employed as an indicator to monitor water and food contamination. Currently, \textit{E. coli} detection methods include plate dilution method \cite{2}, membrane filtration, fluorescence quenching \cite{3}, and the use of quartz crystal microbalance-based sensors \cite{4}. Although all of these methods exhibit relatively high sensitivity and reliability, most of them still have disadvantages. For example, these classic approaches require the cultivation and enrichment of bacteria before detection. Therefore, these traditional approaches cannot enable real-time detection. Moreover, these systems are expensive. Therefore, to rapidly and accurately assess food safety, a fast detection system with compensation must be designed.

Adenosine triphosphate (ATP), a type of nucleotide, is the molecular currency of intracellular energy transfer and is ubiquitous in all cells. Lappalainen et al in 1998 utilized ATP bioluminescence in a hygiene testing system to measure the number of bacteria in fluid milk, and this was measured by measuring the relative amount of light produced\cite{5}. Although this monitoring system was difficult to use on-site, it was proven to be effective. An excellent milestone study by Zhang et al employed bioluminescence to assess food safety \cite{6}, and the quantitative results demonstrated that their method was useful to detect the bacteria with a sensitivity of $3.0 \times 10^2$ CFU mL$^{-1}$. Therefore, this approach is useful to detect small amounts of bacteria in target samples.

Kuang used immunomagnetic separation in tandem with fluorescent probes to detect *Salmonella* with a sensitivity of 500 CFU/mL [9]. In their experiments, two monoclonal antibodies were used to recognize different antigens on the surface of the *Salmonella* and their experiments employing immune-magnetic beads exhibited high enrichment efficiency for *Salmonella* (90%). In 2017, Cheng et al utilized surface-enhanced Raman scattering (SERS) immunoassay and magnetic separation to determine free-to-total prostate-specific antigen ratio to improve the diagnostic performance of prostate cancer [10]. To achieve the concentration chloramphenical effect, they used a magnet to remove the antigen–antibody complex from the supernatant; therefore, SERS measurements became more stable, repeatable, and reliable. However, this technology requires special equipment and expertise; therefore, the technology cannot be widely applied to general laboratory or on-site rapid detection.

Recently, graphene has been receiving more attention as a new bioassay material. In 2004, Novoselov prepared graphene films by mechanical exfoliation and found that it has unique electronic properties [11]. In 2009, Xuesong used graphene chemical vapor deposition to prepare graphene films and also developed a process to transfer graphene film to various substrates, making it possible to prepare transparent graphene electrodes [12]. Bae and Sukang in 2010 used a roll-to-roll production method and wet-chemicals to prepare transparent graphene electrodes [13]. The conductance of transparent graphene electrodes is as low as 125Ω−1, and their optical transmittance is 97.4%, which is better than that of conventional transparent electrodes, such as those fabricated from indium tin oxide.

Traditionally, ATP fluorescence detection system has exhibited low detection accuracy due to the low signal-to-noise ratio and non-specific bacterial recognition. In this study, a rapid detection system prototype based on magnetic separation and ATP enrichment using the force from an electric field was developed. The combination of these techniques not only improves testing accuracy but also enhances measurement efficiency. The system utilizes biotinylated anti-*E. coli* antibodies attached to magnetic nanoparticles prepared with streptavidin-modified magnetic nanoparticles. The detection tube was prepared by chemical vapor deposition (CVD) of graphene [14]. The probe was used to capture the target pathogens, which were then lysed to release ATP. As ATP is negatively charged under weakly alkaline conditions, we were able to use an electric field applied through graphene to enrich the released ATP. The number of bacteria was quantified using light intensity as a metric, as ATP luminescence would be proportional to the number of cells in a sample. As the intensity of ATP fluorescence attenuates over time, we had to improve the experimental method and had to particularly design the subsequent signal processing units to enhance measurement accuracy.

II. Detection principle

ATP is an energy-supplying substance in cells; it is ubiquitous across the cells of various organisms. [15]; ATP comprises adenosine and three phosphate groups. The molecular structure of ATP is presented in Fig. 1.

In our previous study, we prepared immunomagnetic beads (IMBs) [6]. IMBs are magnetic microspheres coated...
with monoclonal antibodies and are frequently utilized for cell sorting, bio-separation, targeted drug delivery, and immunoassays. Such IMBs have advantages such as simple operation, speed, and high efficiency; therefore, they have a wide range of applications in the enrichment and separation of bacteria; these beads do not have the shortcomings of conventional methods such as time-consuming techniques and pre-enrichment requirement [17]. Nanoprobes are ultrasmall biosensors that can detect trace levels of bacteria and whose dimensions are in the nanometer range. In this manuscript, we present the fabrication of IMBs of intermediate size (120–200 nm). Magnetic nanoparticles can be collected by the application of a magnetic field; this property can be exploited to isolate bacteria by their specific binding to nanoparticles. Therefore, IMBs were prepared by modifying their surfaces with streptavidin while modifying E. coli-specific antibodies with biotin and then mixing the two together [18, 19]. Thus, the material could be used to specifically isolate or enrich E. coli using a magnetic field (Fig. 2).

FIGURE 2. Nano gold probe sensor for the capture of E. coli

Immunomagnetic nanoprobe is prepared by combining magnetic nanoparticles with specific antibody to isolate E. coli. Magnetic nanoparticles can be enriched by magnetic field.

Flexible transparent electrode materials have high transparency, light weight, and good flexibility; these electrodes can be prepared in large quantities and in a cost-effective manner; therefore, flexible transparent electrodes are widely used in electronic, optoelectronic, and energy storage equipment [20]. Currently, indium tin oxide (ITO) is widely used in the manufacture of optoelectronic equipment because of its good conductivity and high visible light transmittance[21]. However, ITO has some limitations that prevent its further development. In particular, there is no good solution to the deterioration of its electrical properties upon bending or stretching [22].

Graphene has a mono-layered two-dimensional hexagonal lattice structure made up of unique SP2 hybridized carbon atoms [23]. It possesses excellent characteristics, such as good conductivity [24], light transmittance, excellent mechanical properties and electron mobility at room temperature, a large specific surface area, and high conductivity. Therefore, graphene and its chemically modified derivatives are ideal novel electrode materials and can be used for rapid detection of electroactive molecules in and environment samples [25, 26].

III. Experimental instrument

An H5773-02 type photomultiplier tube (PMT) from Hamamatsu Photonics of Japan was used as the main signal receiver. It detects wavelengths in the range of 300–850 nm, encompassing the luminescent wavelength range of 450–490 nm [27]. Pi-102 (Hygiena, England) was used as a reference. F-4500 fluorescence spectrophotometer (Hitachi, Japan) was used to obtain values for RLU. ADUC834 (Analog Devices, America), which integrates a wealth of on-chip resources, served as a microcontroller [28]. It has two independent ADCs. The primary ADC has a 24-bit resolution, whereas auxiliary ADC has a 16-bit resolution. Therefore, it can greatly simplify system design.

IV. Testing system design

The system mainly consists of optical detection unit and data processing unit as shown in Fig. 3. The optical detection unit is composed of automated filling-up unit, enrichment unit (Reactor #1), and fluorescence reaction units (Reactor #2). The reaction and light detection are carried out in a totally enclosed container. Light trapping is done by the PMT which functions by means of photoelectric conversion. The calibration unit is used to detect the degree of light attenuation; compensation is according to the degree of light attenuation. As the input signal is somewhat weak, system noise is inevitable; therefore, a filter circuit is added in the design to decrease external interference to meet the demands of the system.

FIGURE 3. Block diagram of the prototype detector

The enrichment unit (Reactor #1) uses nanoprobes to capture bacterial pathogens. Under the optimal conditions, the magnetic separation process is described as follows. After
washing with 150 μL of phosphate-buffered saline (PBS) and magnetic recovery, the IMBs are ready to isolate bacteria. Fifty μL of the IMBs and *E. coli* solution are mixed and incubated for 10 min to allow the microbes to bind to the nanopores. Then, the nanopores are washed thrice with 50 μL cleaning solution (PBS containing 0.05% Tween-20) and the supernatant is discarded after magnetic separation. Nanoparticle enrichment is performed using a magnetic rack.

The fluorescent reaction unit (Reactor #2) contains a detection tube with a graphite layer attached to the bottom and a PMT. Figure 4 shows the preparation of the detection tube. The graphene film was developed on a copper foil (thickness 25 mm, purity 99.8%) by CVD at 1000°C with H₂ and CH₄. Poly methyl methacrylate (PMMA) was then spin-coated on the graphene surface and baked for 1 min at 120°C. The PMMA/graphene/Cu assembly was then placed in a beaker containing ammonium persulfate solution (16g in 1000 mL of deionized [DI] water) until the entire Cu foil layer was etched away. The PMMA/graphene film was rinsed three times with DI water and then floated in a beaker. Next, the film was carefully picked up with tweezers and placed at the bottom of the tube, taking care to minimize wrinkles. Finally, the tube was immersed in an acetone bath for 30 min to remove the PMMA, and the graphene sheet was then connected to the top electrode. The PMT can be placed under the detection tube because graphene exhibits good transmittance. The results show that ATP fluorescence is strong under weakly alkaline conditions, while ATP is negatively charged in weakly alkaline conditions. In the detection process, 30 μL of enriched *E. coli* suspension is added to the graphene detection tube and the 270 μL of the detection reagent is accurately injected using a peristaltic pump. Next, an electric field is applied to the detection tube by connecting a graphene electrode to the upper electrode via a stable current source. Automatic reading of optical signal was taken after 60 seconds. Under the action of electric field, ATP is concentrated at the bottom of the detection tube and the oxidation of fluorescein accelerates; thus, the fluorescence is concentrated and the stability of the system is enhanced.

As shown in Fig. 5, the automated filling-up unit allows the system able to select reagents and control the amount of the reagents automatically dispensed. Fine lines represent a single tube, whereas the thick line represents multiple tubes. Different containers contain different reagents.

![Figure 4. Preparation of the graphene detection tube](image)

**FIGURE 4. Preparation of the graphene detection tube**

The purpose of this module is to provide rapid and automatic filling-up. Furthermore, it can also obtain the value of relative luminescence intensity. The detecting time is controlled within 20 min. To improve the efficiency and accuracy of the system, a self-cleaning function is incorporated into the design so that the system is automatically cleaned between tests. Waste liquids from the cleaning process are directed to a waste receptacle. The peristaltic pump is controlled by the MCU and dispenses reagents with an accuracy of 2 mL. The pump tubing is highly elastic and exhibits low adhesiveness and permeability. As shown in Fig. 5, we used a glass cuvette as the reactor to maximize light transmission.

Testing is performed in solution, and the light emitted by ATP luminescence could be absorbed by the solution, which may affect the result. As an internal calibration, two light-emitting diodes with emission wavelengths similar to that of the luminescent light are fixed on both sides of the reactor. The wavelength of diode should be in accordance with the ATP bioluminescence. We must ensure that the luminous intensity to be detected is consistent with that of the light emitted by ATP luminescence. PMT records light intensity of the LEDs both before and after the reagents are pumped into the reactor and the difference between the two optical measurements is considered as the calibration data. To improve accuracy, a steady current source is used to supply power to the LEDs.
FIGURE 6. Signal processing module

A current signal is obtained after the PMT completes photoelectric conversion. It is converted to an analog voltage signal through integrated operational amplifier circuit. The ADUC834 then converts this analog output signal into a digital one, which is then processed by the MCU. Analog to digital conversion requires a reference voltage, which can improve the accuracy of the results. We selected REF192 from ADI Inc as a reference voltage controller and the signal processing block diagram is shown in Fig. 6. The output voltage after processing is approximately 2V, meeting the requirements of the MCU.

V. RESULTS AND DISCUSSION

A. SYSTEM NOISE

In the course of the reaction, as the intensity of ATP fluorescence is extremely weak, system noise has an influence on measurement accuracy. Therefore, we added a filtering circuit to reduce such interference. The first step was to use integral sampling for processing the signal from the PMT. The integral sampler can extract input signals buried in noise, and then use the non-correlation between noise and signal to synchronously accumulate the samples obtained through RC low-pass filter to suppress noise and extract signal. The procedure involves measuring the signal once before and after filtering. Fig. 7 shows the output voltage distribution before and after filtering. Meanwhile, we measure the system noise in a range of −0.2–0.12 mV, (comparing designed system: 0 ± 0.20 mV). It can be observed that the output voltage is more concentrated than before the filtering.

TABLE 1. Binding efficiency of antibody and magnetic nanoparticles

<table>
<thead>
<tr>
<th>A1 (%)</th>
<th>RC (%)</th>
<th>Average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.7</td>
<td>85.9</td>
<td></td>
</tr>
<tr>
<td>5.2</td>
<td>87.0</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>86.4</td>
<td>86.5</td>
</tr>
<tr>
<td>5.3</td>
<td>86.8</td>
<td></td>
</tr>
<tr>
<td>5.6</td>
<td>86.3</td>
<td></td>
</tr>
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</table>

Table 1 shows that the average binding efficiency was 86.5% with a variation of only 0.6% (86.5%−85.9% = 0.6%), indicating consistent and efficient binding of magnetic nanoparticles and antibodies.

B. CAPTURE EFFICIENCY OF MAGNETIC IMMUNOASSAY NANOPARTICLES

It is important that magnetic nanoparticles contain sufficient antibodies to allow them to capture bacteria. The binding efficiency can be measured by the absorbance of the antibody solution before (A₀) and after (A₁) coupling using an ultraviolet spectrophotometer. The binding efficiency (RC) can be calculated using equation (2).

\[ R_c = \left( \frac{A_0 - A_1}{A_0} \right) \times 100 \% \]  

C. ATP fluorescence detection

To assess the kinetic properties of ATP bioluminescence, we tested standard ATP solutions of different concentrations.
(10^{-15}, 10^{-14}, 10^{-13}, 10^{-12} and 10^{-11} \text{ mol/L}) and the results are summarized in Fig. 8.

Luminescence intensity of ATP declined with the time, and there was a linear relationship between ATP concentration and light intensity until the ATP concentration was greater than 10^{-11} \text{ mol/L}. Therefore, we selected samples with concentration of 10^{-13} \text{ mol/L} to examine the relationship between luminescence intensity and time.

As Fig. 9 shows, the output voltage attenuated from a maximum value of 1023.29 mV to 567.39 mV in 100 seconds, and the data fitted the kinetic equation:

\[ A = A_1 \exp \left( -Kt \right) \] [29].

where \( A \) is the luminescence intensity at time \( t \), \( A_1 \) is the luminous intensity at \( t = 0 \), and \( K \) is the attenuation constant. The correlation coefficient of fitted curve was 0.9883. We used this equation to calculate the initial value of luminescence intensity in subsequent experiments.

A standard ATP solution at a concentration of 10^{-13} \text{ mol/L}, with its pH adjusted with hydrochloric acid or sodium hydroxide, was used. Temperature was controlled within a range of 20 ± 2°C. Luminescence intensity was maximum when pH was between 7.5 and 7.9. Intensity approached zero when pH was below 4.0 or greater than 10.0 (Fig. 10). Luminescence is dependent on the enzymatic activity of luciferase, which is inhibited when the pH value is higher or lower. Thus, it is necessary to adjust the pH value to ensure normal physiological activity of the enzyme.

E. SYSTEM STABILITY

A system’s precision is defined by its ability to obtain similar results from samples containing the same amount of analyte. We tested this repeatability using 10 independent samples containing the same numbers of bacteria (numbered 1–10). Coefficient of variation was calculated as CV = SD / Mean ×100% = 4.20% (SD, standard deviation; mean, average value), indicating good test precision (Fig. 11).

F. THE RELATIONSHIP BETWEEN LUMINESCENCE INTENSITY AND BACTERIAL CONCENTRATION

The detection limit is usually defined as the point where the signal is at least three times of the test’s noise level [30]. The standard deviation of the luminescence intensity in this experiment is 150 RLU. In contrast, numerical results obtained using traditional detection method is used as the reference. Standard \textit{E. coli} solutions with concentrations ranging from 10^1 to 10^7 \text{ CFU/mL} were used as sample solutions to test system accuracy. As shown in Fig. 12, there was a linear relationship between the luminescence intensity and the concentration of \textit{E. coli} (10^2–10^6 \text{ CFU/mL}), with a linear correlation coefficient of 0.972. However, when the concentration of \textit{E. coli} was below 10^2 \text{ CFU/mL} or above 10^6 \text{ CFU/mL}, the luminescence intensity was beyond the limit of detection. The enhanced fluorescence intensity improves the detection accuracy of the system; however, it also leads to a slight decrease in the upper limit of inspection.
G. DETECTION ACCURACY

The term absolute error refers to the difference between the measured value and the actual value, which can be defined as absolute error (E) = ABS [log (present method) − log (traditional method)]. When E < 1, the result can be considered accurate [34]. The prototype system was used to test various food products and beverages, and the results were compared with the results of traditional method. Samples were obtained from supermarkets and farmers’ markets including milk, juice, soy milk, beef, pork, fish, wheat, rice, sausage, and meatballs. For solid and semi-solid samples, we placed 25 g into sterile homogenization cups containing 225 mL normal saline and were subjected to homogenization at 10000 rpm for 2 min to produce 1:10 homogenized sample. The liquid sample was collected by suction with a sterile straw and placed in a sterile conical flask containing 225 mL of normal saline and thoroughly shaken to produce a 1:10 dilution of the sample homogenate. This was then inoculated with the *E. coli* and cultured for 24 hours before testing. As shown in Fig.13, measurement accuracy was more than 92%. For example, in the detection of the drinks, the total number is 100 and the number of E < 1 is 99. The results showed that the frozen food obtained lower accuracy because of the lower bacterial content in the samples.

VI. CONCLUSION

This manuscript presents the development of a biochemical system suitable for food safety evaluation. The technique of enrichment of bacteria with magnetic nanoprobes in conjunction with a transparent graphene electrode is used in this system. ATP bioluminescence is used as a proxy for the bacterial content of a sample, making this method more convenient than traditional bacterial detection processes. The system can detect bacterial concentrations in the range of $10^2$–$10^6$ CFU/mL in less than 20 min and its precision has a CV of 4.2%, indicating good reliability and repeatability. These testing results satisfy the requirements of national environmental monitoring department. Furthermore, a good correlation between this method and traditional biological assessment method was observed and the linear coefficient between the two was 0.972. The linear correlation of the method without using GTE enrichment was 0.964. This method of rapid detection provides a better linear correlation with the conventional approaches. This new system is rapid

<table>
<thead>
<tr>
<th>Method</th>
<th>Bacteria</th>
<th>Detection range</th>
<th>Detection time</th>
<th>Linear coefficient</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrochemical method</td>
<td><em>E. coli</em> K1:H7</td>
<td>22 CFU/mL</td>
<td>30 min</td>
<td>0.841</td>
<td>[31]</td>
</tr>
<tr>
<td>Resistive method</td>
<td><em>E. coli</em> Rosetta 2pLysS</td>
<td>$10^6$ CFU/mL</td>
<td>2 h</td>
<td>0.875</td>
<td>[32]</td>
</tr>
<tr>
<td>Magnetic silica nanotubes method</td>
<td><em>S. Typhimurium</em></td>
<td>$10^2$–$10^7$ CFU/mL</td>
<td>30 min</td>
<td>0.901</td>
<td>[33]</td>
</tr>
<tr>
<td>Magnetic nanoprobe-ATP method</td>
<td><em>E. coli</em> O175:H7</td>
<td>$10^2$–$10^8$ CFU/mL</td>
<td>&lt;20 min</td>
<td>0.964</td>
<td>[6]</td>
</tr>
<tr>
<td>Nanoprobe transparent graphene electrode -ATP method</td>
<td><em>E. coli</em> O175:H7</td>
<td>$10^2$–$10^8$ CFU/mL</td>
<td>&lt;20 min</td>
<td>0.972</td>
<td>Present work</td>
</tr>
</tbody>
</table>
and easy to operate, which makes it remarkably useful for field testing. The suitable pH values for the reaction ranged from 7.5 to 7.9; pH directly affects the testing results if it is beyond the suitable values. The system has been designed to contain an automated filling-up unit that can protect the detection from second pollution, and an integrated self-cleaning unit can effectively increase the efficiency.

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