Functional nucleic acids biosensors for living or dead bacteria detection.

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Abstract

As we known, dead and living bacterial infection represents one of the leading causes of disease and death, and as such, bacterial detection is an important step in managing infectious diseases. However, only living bacteria is detected by traditional methods could due to false negative results; especially there are dormant bacteria, which is really a challenge in the early year. In recent years, the development of microbiological testing undergoes a series of progress, from the traditional methods to molecular biological detection, which overcomes the difficulties of detecting the living, dead, and even dormant bacteria. Reviewed its developments, it can be seen that the detection is moving in more rapid, sensitive, precise, integration, and low-cost. Here, a diverse range of dead and living bacteria detection technology including various PCR-based, nanoparticles and spectroscopy technology-Based, dielectrophoresis technology-Based, and flow cytometry-based functional nucleic acids biosensors has been reported. While, a series of prospect to develop the methods for living and dead bacteria detection contact with more new technologies and materials including aptamer approaches, new nanomaterials, and others have been made.

Keywords: Detection; Living bacteria; Dead bacteria; Functional nucleic acids; Biosensors

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Introduction

Microorganisms (bacteria, fungi, protozoa and other species) are ubiquitous and very useful in nutrient cycles, natural decomposition, and various biotechnological processes. However, some of them are harmful and pathogenic. The polluted foods by pathogenic microorganisms may easily make people suffer from poisoning and disease and they can also cause sudden microbial food safety issues. According to the WHO’s statistics, about one-third of people infect foodborne diseases in developed countries every year and foodborne diseases are often the main cause of unnatural death in humans. Bacteria such as Legionella pneumophila attack human macrophages and cause Legionnaires disease, while Campylobacter jejuni and Salmonella typhimurium cause food poisoning. Some bacteria produce toxins, which are secreted to their surroundings to overcome host defenses and are responsible for the symptoms observed in bacterial infections. Bacterial toxins are highly varied, ranging from small molecules such as microcystin-LR, to large proteins such as Shiga toxin and Shiga-like toxin, or even multi-protein mixtures such as Anthrax toxin, which contains a Protective Antigen, an Edema Factor and a Lethal Factor. Fungi also can produce toxic small hydrophobic metabolites known as mycotoxins, such as aflatoxin, ochratoxin A (OTA), zearalenone, fumonisin, and patulin. Protozoa are another large group of microorganisms that are usually harmless; however some protozoa can also cause diseases. For example, Plasmodium falciparum causes malaria, waterborne Giardia, Cryptosporidium, and some Entamoeba species can cause diarrhea.

Foods are polluted by pathogenic microorganisms in the processing of producing, transportation packing and storing. Detection of microorganisms is of great importance for health and safety in many sectors. In clinical settings, the detection of microorganisms is required for diagnostic purposes on patient samples. In food industries, the detection of microorganisms and their toxins is important for food safety and quality control. It is also important in environmental and public sources (e.g. swimming pools, cooling towers, water reservoirs, and soil, etc.) to ensure public safety and to mitigate infectious disease outbreaks. For safety purposes, the maximum level of permitted microbial load in many environmental sources is regulated in several countries. For example, in Singapore, the safety level of Escherichia coli in swimming pools is regulated to be below 1 cfu/100 mL, and the safety level of Legionella bacteria in cooling towers is regulated to be lower than 10 cfu/mL. To control the pollution in the environment of water, air and soil is an instrumental component in understanding and managing risks to human health and the environment and to make a successful treatment for the poisoning and disease by pathogenic infection, the rapid and accurate detection of multiple microorganisms is of great importance in all areas related to health and safety. Thus, there is an expanding need...
for simple, rapid, cost-effective and field portable screening methods for microorganism detection.

Cultural, immune and nucleic acid based methods all have disadvantages and advantages. For example, although the traditional culture approaches only detect living bacteria, they can accurately identify the existing targeted strains in real samples with 3-7 day [1]. The immunological diagnostic methods are quick, but likely to arise nonspecific results [2]. Nucleic acid based technologies, such as PCR, Real-time PCR [3], etc. have been widely applied in microbe detection. What’s more, the nucleic acid molecular biology technology has been widely applied in microbe testing and innovation. Obvious advances in molecular methods have improved microbe analysis specificity and sensitivity while saving time. However, considering these advances, much nucleic acid targets are required to accomplish a detectable signal. Unfortunately, all these approach can not the rapid and simplistic measure to distinguish viable and nonviable bacteria. In a lot of situations, detection of nonviable microbes would give rise to false positive. Viable bacteria can cause food corruption and pathogen city rather than nonviable bacteria. Precisely quantized the amount of living bacterium in food, environment and other fields has been a challenging problem.

Recently, many researchers have pay attention to the identification technology of viable and nonviable bacteria, which overcomes the disadvantage of all bacteria detection by the general molecular method, and has massive potential and important significance in the area of microorganism [4]. Most methods still based on the nucleic acid molecular detection technology that is quickly, specificity, sensitivity, real-time and accuracy, and some methods should use other treatment before the amplification.

In this paper, the development and correlation technique will be introduced in part 1 and 2. In part 3, various functional nucleic acid biosensors for living or dead bacteria detection will be reviewed. At last, we make some conclusion and prospects biosensors for living or dead bacteria detection. This review introduces current condition in this field and inspires researchers to further developing.

**Molecular Biological Detection Technology and Methods in Microorganism**

In recent years, molecular biological techniques have been widely used in microbial detection. The methods based on PCR have been the most common used. PCR technology invented by Kary Mullis in 1985 is one of the most rapid development and popularization of molecular biological techniques. A variety of techniques are derived on the basis of PCR technology, including nested PCR, multiplex PCR, PCR-DGGE and PCR-TGGE, random amplified polymorphic DNA technique, fluorescent quantitative PCR, multiplex fluorescent quantitative PCR, etc. These technologies played important role in microbial diversity analysis, qualitative and quantitative analysis [5].

In addition, the molecule marked method which based on molecular hybridization technology is used in the microbial detection, such as fluorescence in situ hybridization and gene chip technology, etc. The main characteristic of fluorescence in situ hybridization technique is that it combines with the accuracy of molecular biology and visibility of microscope, which can directly observe different microorganism in complex environment. It also can provide much information, such as morphology, quantity and space distribution of bacterial colonies, and cell environmental, to evaluate the microbial community. Because of the sensitive, fast, safe and specificity, fluorescence in situ hybridization technique has been widely used in microbial detection, including activated sludge, agricultural environment, marine environment, biological film, medical diagnosis, soil, and so on [6-8]. Gene chip microarray protocols include a series of steps for DNA purification, specific amplification, fragmentation, labeling, concentration, quantization, dilution, hybridization, washing, imaging, and data analysis. The technology is applied in the fields of analysis and detection of microorganisms [9].

**Functional Nucleic Acids Biosensor for Living or Dead Bacteria Detection**

The state of target cells can be described as "alive" and "viable" and the most important feature of live cells is that live cells are able to grow, divide, metabolize, respire, and reproduce. As a resultmRNA might be a perfect referent of the living cells can be used as an appropriate landmark to detect viable microorganisms by PCR, due to its properties of distinguishing the living or dead cells. This kind of method is to detect animate cell to evaluate accurately the status of bacteria from a sample by identifying the effectiveness of PCR.

**Reverse transcription PCR-based functional nucleic acids biosensors for living or dead bacteria detection**

Reverse transcription-PCR (RT-PCR) can be utilized for analyzing mRNA to monitor gene expression in animate bacteria. RT-PCR had been used to diagnose living Legionella pneumophila and Vibrio cholerae, and proved specific mRNA only in samples that contained viable bacteria detected by culturing. Equally, the viability of heat-killed cell was successfully proved with detecting a heat shock protein mRNA in living Mycobacterium leprae by Patel et al. Similarly, a specific method based on RT-PCR was built by Klein and Juneja to detect the viable L. monocytogenes. However, RT-PCR approach has some limits, which has proved difficult to develop because of the complex assay and a lack of basic information about the significance of detecting mRNA in stressed cells [10]. More recently, a Real-time RT-PCR (RRT-PCR) assay has been developed. And it is an evolution of RT-PCR which has two steps, multiplex assay based on targeted sequences for the detection. RRT-PCR has been successfully utilized to detect various RNA viruses with hydrolysis probes by Holland et al. and Livak et al.

Compared with the traditional approaches, the detection approach as the excellent and wide-utilized technology in the early period is easier, faster, and reproducible. However, there
are some limits such as requiring complex operations including extracting, purifying the mRNA from cell. In addition, there are also some problems that to extract and preserve intact RNA in the physiological condition or the environment condition [11].

Viability PCR-based functional nucleic acids biosensors for living or dead bacteria detection

Real-time PCR (QPCR) combined with viability dyes also is a significant and practical method, which has been widely utilized in different types of food borne pathogens. DNA-binding dyes such as Ethidium Monoazide (EMA) or Propidium Monoazide (PMA) can permeate dead or membrane-compromised cells as pretreatment previous to the QPCR. The novel approach to detect viable and nonviable cells has been introduced coupling PCR or QPCR with DNA-binding dyes named viability PCR (v-PCR).

Ethidium Monoazide Bromide (EMA) is a DNA/RNA intercalating chromophore, which can penetrate damaged cytomembrane and embed in DNA upon photoactivation. As a result, it can inhibit subsequent molecular amplification include PCR, QPCR, LAMP, and others [12]. Shi et al. had optimized the condition of EMA treatment, the level of thermal disinfection and the value of pH and osmotic pressure, and well documented the probability of EMA-QPCR assay [12]. Amplification will be inhibited when EMA enter nonviable bacteria and bind to the nucleic acid. Light exists can promote the combination of EMA and DNA. So, the optimum concentration of EMA is 10 μg ml⁻¹, and photoactivation time is 20 min. Cells in various states can be bound in EMA totally by heating. The optimum treatment condition to yield inactivated cells could be considered at 85°C for 35 min, which could be not detected by EMA-QPCR. High osmotic pressure (≥ 4%) could increase the inhibition of EMA-QPCR, and the inhibitory effect is positively correlated to the osmotic pressure. The counts of cells decreased sharply by EMA-QPCR when the osmotic pressure increased to 8%. Cells were treated in different pH solutions and then subjected to EMA treatment, the Ct values prominently increase from pH 1-5, and inhibitory effect is positively correlated to the osmotic pressure (≥ 4%) could increase the inhibition of EMA-QPCR, and the inhibitory effect is positively correlated to the osmotic pressure. The counts of cells decreased sharply by EMA-QPCR when the osmotic pressure increased to 8%. Cells were treated in different pH solutions and then subjected to EMA treatment, the Ct values prominently increase from pH 1-5, and no difference after pH 5. It also showed that the counts had already decreased significantly at pH 3. The sublethal acidification injury cells could be completely recovered and EMA could not penetrate them with 40 min LB incubation.

EMA is an important dye which can distinguish the living cells or the dead cells according to that if it can enter the cell, which depends on the membrane integrity. EMA-QPCR was reported to be a simple and easy-practical method to distinguish living and dead cells. However, EMA has the limits of low ability to bind the DNA insoluble, and strong ability to penetrate viable cells of other bacterial species. To overcome the difficulties, propidium monoazide (PMA) has been successfully developed to differentiate viable and nonviable bacteria in conjunction with QPCR. PMA is able to enter the membrane of heat-killed bacterial cells and intercalate the DNA or bind to any free DNA in a sample and inhibit the activity of the Taq polymerase [13]. Later, Lee et al. found that following the heat treatment of cell suspensions the surviving populations with the EMA and PMA, the percent of erroneous survival with PMA was higher than with EMA treatment [14]. PMA was also coupled with the multiplex PCR (mPCR) to detect enterotoxin genes (nheA, entFM, hblD, cytK) and emetic toxin (ces) in the viable Bacillus cereus [15]. The PMA had also been combined with 454 pyrosequencing to detect bacteria [15].

In conclusion, V-PCR has showed the great potential for the identification and detection of viable and nonviable microorganisms. Moreover, PMA-QPCR was more widely used in microorganisms than EMA-QPCR. The drawback of v-PCR is that the exclusion of dead cells can be incomplete leading to false-positive signals [16]. New approach improves v-PCR by enabling it to also discriminate between cells with an intact cell membrane and the ability to actively maintain bacterial homeostasis and cells that have an intact membrane but are metabolically inactive [17].

Nuclease PCR-based functional nucleic acids biosensors for living or dead bacteria detection

Microorganism can maintain a low metabolically active for a long time when the environmental condition is harmful. The organism may also remain dormant state that has been named “Viable but Nonculturable” (VNC). These VNC organisms will not grow but can survive in the environment for a few weeks. V-PCR has been increasingly used for detection of viable and nonviable cells, but the hinder still exist in v-PCR, which is the separation of viable and nonviable cells. Therefore, the sample preparation methods were demanded to microorganisms, whether they are living, VNC, or dead. In the QPCR assay, when hybridization occurs, the dyes-labelled probe is cleaved by the 5'-nuclease during the extension, generating increase fluorescence intensity. An approach to the reduction of the background signal generated by DNA in heat-killed bacteria by using external DNases was evaluated [18]. The EVA also could covalently bind to DNA inhibited the 5'-nuclease PCR [19].

The quality of the nucleic acids from the dead cells is influenced by itself and other treatment. The inherent bacterial DNases can affect the half-life of DNA. It is important to confirm the stability of nucleic acids to explain whether the positive PCR signal is root in living cells. The physical difference between viable and nonviable cells has not yet been widely applied in PCR. The nucleic acids in viable cells are protected due to the intact membranes. But the exposure of DNA in nonviable cells is easier impaired by outside contamination. Deoxyribonuclease I (DNase I) is an endonuclease that can cleave single and double stranded DNA. DNase I has been widely used in removing genomic DNA in sample for RNA analyses. Free DNA in nonviable cells should not be protected from the DNase I due to its damaged membrane. In theory, only DNA from viable cells will be present in the template after treated by DNase I for subsequent QPCR analyses. In Villareal’s research, it was reported about a DNase I-and Proteinase K-based treatment protocol developed and optimized for the detection, characterization, and analysis of live populations of bacteria present in drinking water biofilms [20].
PCR combined with different kind of nucleases as a powerful tool can distinguish the living, dead, and even “viable but nonculturable” microorganisms, which overcome the difficulties of analyzing VNC organisms and improve the performance of PCR only.

Nanoparticles and spectroscopy technology-based functional nucleic acids biosensors for living or dead bacteria detection

An important challenge in bacteriology is to identify whether a cell is living or dead especially when bacteria are starved, or in a severe environment. The bacterial in this condition will be very difficult to distinguish a cell because it is alive but unable to grow or dead. Methods based on the nucleic acid dyes to penetrate the incomplete membrane have been to overcome this challenge. Nevertheless, the problem is still exist, a new approach to probe viable and nonviable cells, using nanoparticles and spectroscopy technology have been developed. As Atomic Force Microscopy (AFM) imaging of viable cell becomes more commonplace, and AFM does not affect cell viability during and after the process. At the same time, there are plenty of probe-membrane interactions that lead to transfer of membrane components to the probe [21]. Later, AFM has been explored viable cells at the nanoscale. It probed the mechanical properties of viable and nonviable cells via AFM indentation experiments. And it immobilized a single cell onto a surface for AFM, without the need of a chemical fixation [22].

Both Fourier Transform Infrared (FTIR) and Raman spectroscopy have been applied to monitor a biologically active by surface reactions. The Raman micro-spectrometer has also been used to in situ rapid discriminating the viable cells, when 58S substrates, 45S bioglass, and bioinert silica were modified on the viable cells. Viable cells could be good monitored by a high power 785 nm laser, without cell damage. And cell death will induce strong changes in the Raman signature (1000-1150 cm\(^{-1}\)) [23]. Both Raman and Surface-Enhanced Raman Scattering (SERS) display highly specific spectral fingerprints of bacteria. Nevertheless, the sensitive of SERS mapping should be developed by novel nanostructures. The novel SERS mapping could obtain a visual signal of live and dead bacteria had been reported to recognize live and dead bacteria with silver nanoparticles (Bacteria@AgNPs) [24].

Recently, research had shown the advantage of Laser-Induced Breakdown Spectroscopy (LIBS) with nanosecond or femtosecond laser pulses to distinguish microorganism. *Escherichia coli* are genetically well characterized, which has divergent cations in outer membrane. And it also can be an appropriate bacteria model to simulate basic states of alive and dead [25].

With the development of more and more nanoparticles technologies, the approaches combined with nanoparticles attracted attention of scientist increasingly. Due to their unique physical and chemical properties, nano-materials have been extensively used to develop biosensors for rapid detection of microorganisms as target analytes compared with ordinary materials.

**Dielectrophoresis technology-based functional nucleic acids biosensors for living or dead bacteria detection**

Dielectrophoresis (DEP) had been applied to characterizing viable and non-viable cells by non-uniform AC electric fields. Viable and nonviable cells have different frequency responses. Microelectrodes can be used to make positive and negative DEP that has been used to distinguish *Saccharomyces cerevisiae* [26]. Hydrodynamic DEP could be made in a high-throughput chip for separation of bacteria. The chip has three planar electrodes in every separation channel. In this channel, the target and non-target bacteria will move away or remain stay in the central streamline, respectively [27].

Insulator-Based Dielectrophoresis (iDEP) is a new kind of DEP. The innovation of the iDEP is the use of insulators, avoiding the problems of electrodes. Viable and nonviable cells cannot be distinguished by electrokinetic mobility, but can be distinguished by dielectrophoretic mobility. Nonviable cells have lower dielectrophoretic mobility than viable cells. The iDEP showed potency to separate viable and nonviable cells simultaneously for bacterial analysis [28]. The iDEP trapping microchip with the open-top microstructures are fit for capturing cells. At the frequency of 1 kHz, nonviable cells can be trapped from viable cells [29]. Contactless dielectrophoresis (cDEP) is a developed technology of cell screen. Viable cells can be screened from dead cells use their electrical characteristic. The cDEP method unites with other advanced technologies can be made in lab-on-a-chip systems for identifying cells [30]. Reservoir-based dielectrophoresis (rDEP) has special advantages as compared to DEP such as the occupation of zero channel space and the elimination of any mechanical or electrical parts inside microchannels. This approach had been used to selectively trap dead yeast cells and continuously separate them from live ones. It also can be combined with other components into lab-on-a-chip devices for applications to biomedical diagnostics [31].

Dielectrophoresis is one of the most effective and widely used techniques for manipulating, separating, sorting, and identifying biological cells. It is utilized to detect viable and nonviable microorganisms and various kinds of dielectrophoresis and devices have been developed.

**Flow cytometry-based functional nucleic acids biosensors for living or dead bacteria detection**

Flow Cytometry (FCM) as a conventional means for cellular biology has been used since the 1970s. The FCM is a potential method because of its high-throughput capacity and the ability for differentiating single-cell. Some cells may show metabolic activity and capable to grow, while some may have intact membranes but are no capable to grow. Other cells may be dead or dormant, have injured membranes, and no capable to replication. Different character between viable, dormant, and nonviable cells is the key to different by FCM. Different character such as cytoplasmic and membrane integrity,
intracellular enzyme activity, respiration rates and nucleic acid content, all can be the key point. Nucleic acid binding dyes have been widely used to analyze cell viability. The most common being SYTO dyes and propidium iodide (PI). In addition, the 3, 3′-dihexyloxacarbocyanine (DiOC6 (3)) was used to determine the membrane potential, the 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) dye was used to discriminate cell respiring, carboxyfluorescein diacetate (cFDA) was used to monitor esterase activity [32]. When the cells were simultaneously treated with cFDA and PI, flow cytometry and cell sorting revealed a striking physiological heterogeneity within the stressed Bifidobacterium population. In situ assessment of the physiological activity of stressed Bifidobacteria using multiparameter flow cytometry and cell sorting may provide a powerful and sensitive tool for assessment of the viability and stability of probiotics [33]. The LIVE/DEAD BacLight kit is more and more popular among the various fields of bacteria. It can be combined with FCM and used to determine the membrane potential, the 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) dye was used to discriminate cell respiring, carboxyfluorescein diacetate (cFDA) was used to monitor esterase activity [32]. When the cells were simultaneously treated with cFDA and PI, flow cytometry and cell sorting revealed a striking physiological heterogeneity within the stressed Bifidobacterium population.

Flow cytometry utilizes the difference features of cells to characterize viable, dormant, and nonviable cells. The various nucleic acid binding dyes can reflect the different cell membrane, esterase activity respiration rates, nucleic acid content, and so forth respectively. Flow cytometry has good prospects for development with its ability to differentiate single-cell.

**Biosensors for living or dead bacteria detection**

There are three types of sensors which are physical sensors, chemical sensors, and biosensors. Biosensors bear remarkable advantage, such as specificity, sensitivity, real-time sensing, time-efficiency, in-situ monitoring, and so forth. Biosensors collaborated with nanomaterials, has been applied in the detection of active molecule, whole cells, and other targets. There are various classifications of biosensors, and one of them is electrochemical biosensors. The electrochemical biosensors also can be classified as potentiometric, impedimetric, amperometric, and conductimetric [35]. A highly specific and sensitive RNA biosensor had been developed to detecting viable *Escherichia coli* which were identified and quantified by mRNA (cplB) gene with NASBA-based biosensor [36]. In another research, an impedance biosensor, double Interdigitated Array Microelectrodes (IAM) have been developed, which is based flow cell. If bacterial grew, impedance would be measured inside the flow cell. Single IAM-based flow cell was lower sensitive than double IAM-based flow cell in the detection of *E. coli* O157:H7. Therefore, the double IAM-based flow cell can be good used to sensitively detect viable bacterial [37]. A microfluidic platform using EMA had been used to detect and type viable and nonviable bacteria and it is the first time to detect and type viable bacteria continuously on the same microfluidic platform [38].

Lateral flow tests (LFTs) or test strips that are point-of-care biosensors based on paper, have been developed as a diagnostic tool in the laboratory. LFTs are usually labelled with colloidal gold, latex, carbon, up-converting phosphorus, single-strand nucleic acid and so on. The most common state is LFT labeled with colloidal gold particles. Fluorescent immunoliposomes have more sensitivity than color labels for visual detection. Up-converting phosphorus may be 10- to 100-fold more sensitive than colored latex beads and colloidal gold. And it is proved that europium chelate-loaded silica nanoparticles improve sensitivity in LFTs compared with colloidal gold. LFTs present enormous potential and have been considered as an available method for field tests of bacteria, such as *Listeria, E. coli, S. aureus, Salmonella, Campylobacter, and Clostridium* [39]. For example, adenosine triphosphate (ATP)-based bioluminescence is the combination of LFT and mutant firefly luciferase, which is adopted for the assay of Salmonella enteritidis. In this approach, viable *S. enteritidis* could form a Salmonella-antibody-gold complex, which would be captured at the test line and lysed by heat treatment. At the same time, the viable cells would release ATP, measured by mutant luciferase [40].

Biosensors combined nucleic acids and other techniques are utilized widely in living or dead microorganism detection with high sensitivity and short detection time. For example, viable *L. monocytogenes* can be detected within several hours including RNA extraction, amplification, and visualization and the detection limit is as low as 20 CFU/mL [41].

**Conclusions and Prospect**

As mentioned above, viable and nonviable detection is of vital importance. Analysis of viable and nonviable microorganisms has been researched and developed many years and remains a significant challenge. In recent years, the development of microbiological testing undergoes a series of progress, from the traditional methods, including microbial cultivation, physiological and biochemical testing, instrument analysis, immunology, to molecular biological detection. And the nucleic acid molecular detection technology plays more and more important role which traditional methods cannot match with in the microbial detection with virtues of quickly, specificity, low-cost, real-time, and accuracy in recently years. For example, nucleic acids based technologies can detect as low as 20 CFU/mL within several hours while culturing methods cost several days in general. There are no needs of instruments for nucleic acid based method which can decrease the cost of detection greatly.

Although a lot of methods have been developed, there is great potential for promotion. In recent years, nucleic acid dyes are combined with molecular amplification to distinguish viable, dormant nonviable bacteria and PCR are most commonly used. Other novel amplification methods can be further developed. When these techniques are used together, their combined strength can help overcome the weakness of the identification and detection methods of viable and nonviable bacteria, and extend a new field. In addition, samples like food, groundwater or soil have many complex factors such as turbidity, substances
reacting with dyes and high concentration of dead microorganisms, which affect analysis and results of post-processing. The special aptamer can associate with the target bacteria to form a complex completely undisturbed. More aptamer platform can be developed for high sensitivity. Besides, nano-materials with various properties and rapid development can facilitate the sensitivity and specificity to some extent. Thus, it has potential to apply novel nanoparticles widely in more viable and nonviable analysis methods. Last but not least, the principles of nucleic acid detection such as mRNA can be further researched and explored to increase sensitivity and shorten detection time basically.

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