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Label-Free and Abasic Sites Containing DNA Probes for Selective Recognition of *Staphylococcus aureus*

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The purpose of this study was to develop novel methods which could rapidly select and recognite *Staphylococcus aureus*. This study described two label-free methods using specific DNA sequences and 2-amino-5,6,7-trimethyl-1,8-naphthyridine (ATMND) to detect *Staphylococcus aureus*. In this assay, complementary double strands DNA probe and stem-loop structure probe can hybridize with a target DNA, so the DNA strand undergoes a conformational change to adopt an open state, resulting in restoration of the fluorescence intensity due to the release of the ATMND from the dSpacer site. The fluorescent intensity is related to the existence of *Staphylococcus aureus*. The study results demonstrated the both two methods could induce specific fluorescent signal after the *Staphylococcus aureus* DNA was added compared with other negative control strain DNA. Finally, this test provided methodological exploration for rapid and sensitive *Staphylococcus Aureus* identification by using Label-Free and Abasic Sites Containing DNA Probes.

Key words: Label-Free, ATMND, DNA Probes, *Staphylococcus aureus*.

In recent years, diseases caused by pathogenic bacteria have attracted widespread attention. Thousands of pathogenic bacteria have been identified as harmful to humans and animals. Certain bacteria act as pathogens and cause mild infections like sore throat, pneumonia etc., but few types of pathogens also can cause shock and even death.

Staphylococcus aureus is a kind of gram-positive coccus, which can cause a series of illnesses, from mild skin infections, including: impetigo, cellulitis folliculitis, to severe infected diseases such as pneumonia, meningitis, endocarditis, toxic shock syndrome (TSS) and sepsis. *Staphylococcus aureus* can be frequently found on the skin and nasal passages. It is reported that 20% of the population are long-term carriers of *S.aureus*[1]. Current conventional *Staphylococcus aureus* detection methods mainly rely on culture, which at least should cost 3 to 5 days and demand high clean environment.

In recent years, several new methods have been developed for identification and discrimination of *S. aureus* in medical field and foods, such as, gold nanoparticle-based immuno chromatographic assay[2], latex agglutination test[3], surface plasmon resonance biosensors[4], impedance electrochemical sensor[5-10] and Polymerase chain reaction-enzyme immunoassays

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to detect *Staphylococcus aureus* enterotoxin A and B genes[11]. Although these methods are more rapid than traditional culture and the theory are feasible and excellent, reproducibility of result prevent the development of the actual samples in the real world. Therefore, it is urgent to develop new methods for rapid *Staphylococcus aureus* detection, which can really be used in practical field and easy to be accepted by laboratory.

Different bacteria have different specific DNA fragment. Based on the recovery of the specific DNA, amplification of target nucleotide sequences has been successfully used to detect microorganisms. Molecular beacon is a special DNA sequence structure. Conventional DNA molecular beacons are single-stranded DNA with stem-loop structures. The loop is complementary to a target DNA, and the stem consists of two complementary sequences. A fluorophoreis attached to the end of single-strand DNA and a quencher combined to another. In the absence of the target DNA state, the molecular beacon is stem-loop structure. The fluorophore and the quencher's close proximity, causes fluorescence is quenched. Those structures are unique. However, these labeling with fluorophore and quencher are expensive. 2-amino-5,6,7-trimethyl-1,8-naphthyridine(ATMND) is an extrinsic fluorophore, which can bind to vacant site and the ATMND's fluorescence can be quenched by C opposite site, so we adopt the stem-loop structure saiming at bacterial specific target genes and develop a novel label-free system to detect fluorescence. The label-free system combined with ATMND have been successfully developed for adenosine, Pb^{2+},UO_2^{2+},Hg^{2+}detection[12-14]. The double strands DNA probe and stem-loop structure have been created and used as versatile tools to detect targets in molecular diagnosis. Conventional double strands DNA probe and stem-loop structure require covalent coupling of fluorophore and quencher to either the end or the internal site of the same DNA strand. The interaction between double strands DNA probe and stem-loop structure with target DNA induces the separation of the fluorophore and the quencher, causing an observable increase in fluorescence[15-24]. Relying on the dSpacer label-free system, using ATMND can achieve the same effect and need less fluorescent cost and lower environmental requirements. At the same time, the novel method, which we developed, can shorten the detection time compared with the traditional methods obviously. The most important is bacteria detection depends on ATMND and the special DNA structures have never been reported yet.

Here, we designed two rapid *Staphylococcus aureus* detection systems according to DNA hybridization, based on standard complementary double strands DNA measure and stem-loop structures measure with ATMND. In the absence of target DNA, double strands DNA sequences binding with extrinsic fluorophore ATMND, as is shown in Fig.1 and Fig.4. Since ATMND can automatically insert to dSpacer site and be quenched by the cytosine, when hybridization with a target DNA, the DNA strand undergoes a conformational change to adopt an open state, resulting in recovery of the fluorescence intensity due to the release of the ATMND from the abasic sites. The dSpacer label-free systems using ATMND have been reported in metal ions and single-nucleotide, but bacteria, especially real samples have never been studied. Herein, the unique stem-loop structure with dSpacer and the corresponding based on cytosine was adopted. Therefore, fluorescent intensity could indicate the existence of bacteria. Moreover, we demonstrated the application of the stem-loop structure and double strands DNA probe sequences of real strain sample DNA and evaluated the feasibility of two novel methods.

MATERIALS AND METHODS

Reagents

Fluorophore 2-amino-5,6,7-trimenthyl-1,8-naphthyridine(ATMND) was purchased from Ryan Scientific Inc (SC,USA). All DNA samples used in the work were synthesized by Shanghai Sangon Biotechnology Co. Ltd (Shanghai, China) and the sequences are shown in Table 1 (X represents dSpacer). The Bacterial DNA extraction kit was purchased from Ding Guo Chang Sheng Biotech. Co. Ltd (Beijing,China). The other reagents were commercially available analytical grade and were used without further purification. Double distilled water was used throughout the whole experiment.

Strains and Culture Medium.

All positive control strains and negative control strains are as follows: *Staphylococcus*

aureus (ATCC6538), *Escherichia coli* (ATCC 25922), Monocytes Listeria (CMCC(B) 54002), *Enterobacter sakazakii* (ATCC2900), *Pseudomonas aeruginosa* (ATCC27853), *Salmonella* (CMCC(B) 50115). All strains were maintained in semisolid Guangdong Huankai Microbial SCI. &THCH, Co., Ltd agar. (Guangdong, China) at -4°C and were cultured and checked for purity by Guangdong Huankai Microbial SCI. & THCH, Co. Ltd Brain Heart Infusion Broth or blood Agar Plate (Guangdong. China). All preparations of experimental suspension work were carried out in a biosafety cabinet. Aseptic technique was used during the whole process. All bacterial waste was sterilized at temperature of 121°C for 20 minutes prior to disposal.

Fluorescence Measurements

In standard complementary double strands DNA measurement, 14μL mixture (100μL 10μM probe DNA, 100μL 10μM complementary DNA and 200μL buffer), ATMND 10μL(1μM) and different volume in the range of 2-60μL target DNA (10μM) were added sequentially into a microcentrifuge tube. After vortexing, the mixture solutions were annealed after heated by Mastercycler (Eppedorf, Germany) at 70°C for 10min and gradually cooled to 5°C in 30min, and then the mixture was transferred to a quartz cuvette in the fluorimeter with constant temperature control at 5!. After 5 min, the fluorescence intensity at ex/em=358/405nm was recorded by fluoro spectrophotometer (F7000 Hitachi Japan). And then, 30μL (20ng/μL) *Enterobacter sakazakii, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus* real sample extraction DNA were added into the mixture instead of target DNA to detect by fluoro spectrophotometer as above.

In standard stem-loop structure measurement, 300μL buffer (10 mM HEPES pH7.0, 100 mMNaCl and 1mM EDTA), 20μL ATMND (1μM), 30μL beacon DNA (10μM) and different volume in the range of 5-40 μL target DNA (10μM) were added sequentially into a microcentrifuge tube. The measurement conditions and experimental procedures were same with the complementary double strands DNA measurement mentioned above. The 30μL (20ng/μL) positive and negative control strains DNA finally be used as the method validation.

RESULTS

Design of complementary double strands DNA structure containing a dSpacerabasic site

We designed a unique label-free complementary double strands DNA probemethod with ATMND to *Staphylococcus aureus*. To achieve this goal, the study was to transform the structural switching of the target DNA upon target-binding into fluorescence enhancement. We can see clearly from the Fig.1, the ATMND was released when the complementary functional target DNA was added to mixture. Fluorescent signal, consequently, enhances.

We can see the intensity of fluorescent signal raised after the target DNA has been added in the range of 2-60 μL from the Fig.2. In addition, real sample DNA were also investigated under the same conditions. The negative control bacteria, such as *Escherichia coli, Monocytes Listeria, Enterobacter sakazakii, Pseudomonas aeruginosa* and *Salmonella*, could induce little fluorescence enhancement response, whereas *Staphylococcus aureus* could bring strong fluorescence enhancement response after the DNA were added from the Fig.3.

Design of stem-loop structure containing a dSpacerabasic site

Encouraged by the above *Staphylococcus aureus* complementary double strands DNA with the ATMND method result, we developed a unique loop-stem of several base pairs with a dSpacer site, as shown in Fig.4. This method

Table 1. Sequences of oligonucleotides used in this work

Name	Sequences
Target DNA	5'- TGGACGTGGCTTAGCGTATATTTATGCTGATG - 3'
Stem-loop structure	5'-CATCAGCATAAATATACGCTAAGCCACGTCCATATXCTGATG- 3'
Probe (25)	5'-CATCAGCATAAATATACGCTAAGCCACGTCCA - 3'
Complementary DNA	5'-TGGACGTGXCTTAGCGTATATTTATGCTGATG - 3'

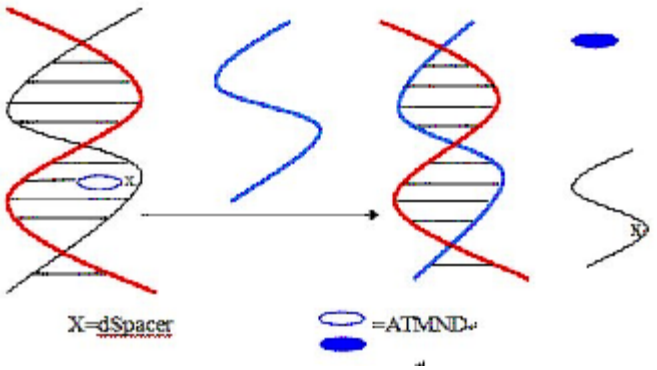


Fig. 1. Scheme of *Staphylococcus aureus* detection using complementary DNA with dSpacer site

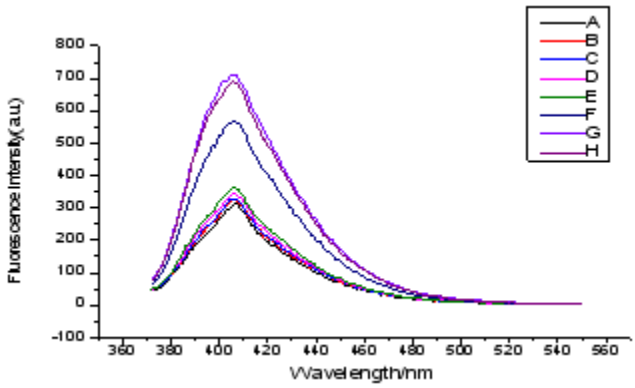
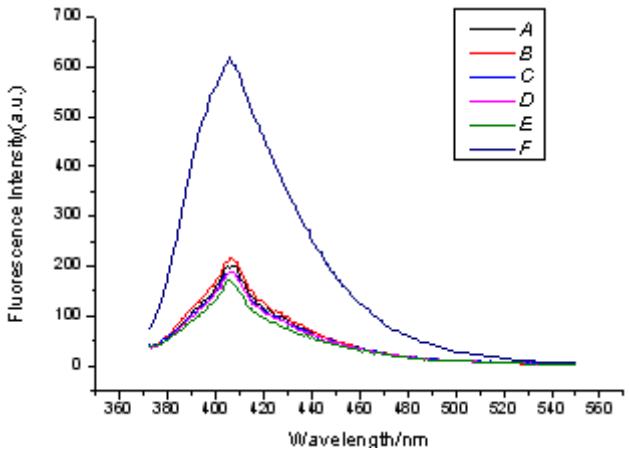
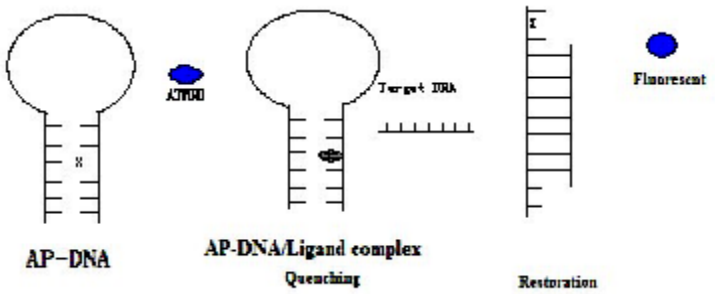


Fig. 2. Relationship between fluorescence enhancement rate and target DNA concentrations. A:1μLATMND, 14μL mixture, 5μL targe DNA; B:10μL ATMND, 14μL mixture,10μLtargeDNA; C:1μLATMND, 14μLmixture, 15μLtargeDNA; D:1μLATMND,14μL mixture, 20μL targe DNA;E: 1μLATMND, 14μL mixture, 30μL targe DNA; F: 1μLATMND, 14μL mixture, 40μL targe DNA; G: 1μLATMND, 14μL mixture, 50μL targe DNA; H: 1μLATMND, 14μL mixture, 60μL targe DNA.



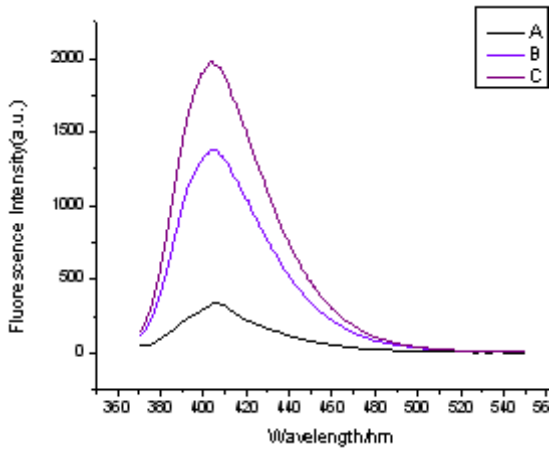
A:*Enterobacter sakazakii*; B:*Escherichia coli*; C:*Monocytes Listeria*; D:*Pseudomonas aeruginosa*; E:*Salmonella*; F: *Staphylococcus aureus.*

Fig. 3. Fluorescence spectra of complementary DNA/ATMND in the presence of real sample DNA



AP :apyrimidinic or apurinic sites,

Fig. 4. Scheme of *Staphylococcus aureus* detection using stem-loop structure probe with dSpacer site methond



A:ATMND;B:ATMND, Stem-loop structure probe,buffer,Target DNA; C:ATMND, stem-loop structure probe, buffer

Fig. 5. Fluorescence spectra of stem-loop structure probe /ATMND in the presence of target DNA

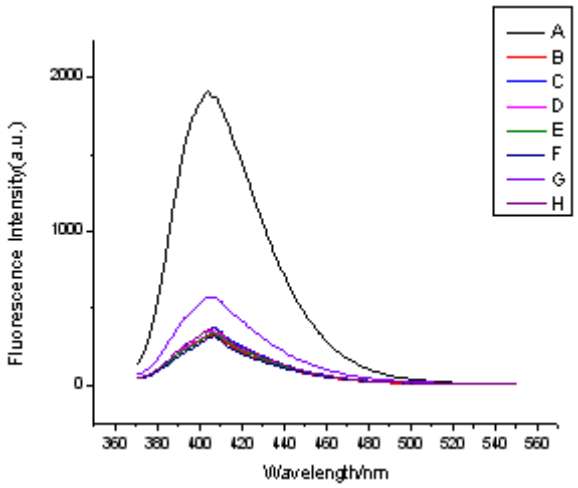


Fig.6. Fluorescence spectra of stem-loop structure prob/ATMND in the presence of real sample DNA. A:ATMND;G:ATMND, stem-loop structure prob,buffer, *Staphylococcus aureus* DNA; B: ATMND, stem-loop structure prob, buffer; C: ATMND, stem-loop structure prob, *Enterobacter sakazakii* DNA D: ATMND, stem-loop structure prob, buffer, *Monocytes Listeria* DNA; E: ATMND, stem-loop structure prob,buffer, *Escherichia coli* DNA; F: ATMND, stem-loop structure prob, buffer, *Salmonella* DNA; H: ATMND, stem-loop structure prob, buffer, *Pseudomonas aeruginosa* DNA.

compared with the above complementary double strands DNA measure is more unique and sensitive. To validate the feasibility of this method, the effect of on the fluorescence response before and after adding target DNA were detected. Since the location of ATMND on the DNA was controllable in the label-free method, an abasic site-containing stem-loop structure was designed to hybridize to the *Staphylococcus aureus* target DNA and real sample DNA. In the absence of the target DNA, ATMND bound strongly to the abasic site in the duplex region, resulting in suppression of its fluorescent signal. When the *Staphylococcus aureus* target DNA was added in the solution, the quenched fluorescence of ATMND was recovered (Fig.5).

In addition, this method maintained excellent selectivity over other negative controls, compared with *Escherichia coli, Monocytes listeria, Enterobacter sakazakii, Pseudomonas aeruginosa* and *Salmonella*. From the Fig.6, *Staphylococcus aureus* DNA (G) had strongly fluorescent signal recovering compared with other negative bacteria (B, C, D, E, F, H). Consequently, *Staphylococcus aureus* could be effectively detected through the fluorescence enhancement strategy.

DISCUSSION

The two novel designs used in this study are based on a special abasic site called dSpacer for nucleic recognition. When complementary double strands DNA probe and stem-loop structure hybridize with a target DNA, the DNA strand undergoes a conformational change to adopt an open state, resulting in restoration of the fluorescence intensity due to the release of the ATMND from the dSpacer site. The fluorescent intensity is related to the existence of *Staphylococcus aureus.*

In summary, we have developed two novel methods to detect *Staphylococcus aureus*. The two methods depend on ATMND combined with characteristic of fluorescence recovery and the results demonstrated the both two methods were able to induce specific fluorescent signal after the *Staphylococcus aureus* DNA was added compared with other negative control strain DNA. On the other *hand*, the assays were sensitive, specific and rapid to *Staphylococcus aureus*. Compared with conventional chemical fluorescent label, these novel methods adopt ATMND with low-cost and unique detection advantages. We hope these methods could be applied to detect other pathogen or viruses that cause illness. And we are going to further apply the method in more realistic and complex situations.

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