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The use of a 2-aminopurine-containing split G-quadruplex for sequence-specific DNA detection

Sung Hyun Hwang[a], Woo Young Kwon[a], Hyunmin Eun[a], Sehan Jeong[a], Jun Seok Park[b], Kwang Jin Kim[c], Hyung Joo Kim[a], Sang Hyun Lee[a], Kyungmoon Park[d], Jeong-Jun Yoon[e], Yung-Hun Yang[a] and Ki Soo Park[a]

[a]Department of Biological Engineering, College of Engineering, Konkuk University, Seoul, Republic of Korea; [b]Colorectal Cancer Center, Kyungpook National University Chilgok Hospital, School of Medicine, Kyungpook National University, Daegu, Republic of Korea; [c]Urban Agriculture Research Division, National Institute of Horticultural and Herbal Science, Rural Development Administration, Wanju, Republic of Korea; [d]Department of Biological and Chemical Engineering, Hongik University, Sejong City, Republic of Korea; [e]Intelligent Sustainable Materials R&D Group, Korea Institute of Industrial Technology (KITECH), Cheonan-si, Republic of Korea

ABSTRACT

A simple, sequence-specific DNA detection method, utilizing a fluorescent 2-aminopurine (2-AP) nucleobase analogue-containing split G-quadruplex as the key detection component, is described. In the sensor, the 2-AP-containing G-quadruplex is split into two segments and linked to a target-specific overhang sequence. The separate G-quadruplex sequences form an active G-quadruplex structure only in the presence of a complementary target DNA, which leads to a significant increase in the intensity of fluorescence from the 2-AP fluorophore. This simple, one-step, homogenous assay was successfully employed to detect target DNA with a high selectivity. In addition, the practical applicability of the detection method was demonstrated by its use in analyzing target DNAs in human serum. To the best of our knowledge, this is the first time that an investigation was carried out in which a fluorescent nucleobase analogue was incorporated into a split G-quadruplex structure and this structure was utilized as the foundation for a specific DNA sensor.

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Biosensor; fluorescent nucleobase analogue; molecular diagnostics; sequence-specific DNA detection; split G-quadruplex

Introduction

The detection of DNA is important in the area of clinical diagnostics, biomedicine and forensic science [1]. Since all of organisms have their own unique genetic information, the specific DNA sequences can serve as a potential identification marker. Until now, different methods such as electrochemistry, colorimetry and fluorometry have been developed to detect DNA. Among them, fluorometry has received the special attention because of its simplicity and high sensitivity [2,3].

Fluorescent nucleobase analogues not only retain the chemical and biological properties of natural nucleobases, such as base pairing and enzyme binding, but they also possess conformation-dependent fluorescence efficiencies [4–6]. A representative example of this type of nucleobase is 2-aminopurine (2-AP), which displays efficient fluorescence emission when incorporated in a single-stranded DNA [7,8]. Moreover, the fluorescence of 2-AP is strongly quenched when present in double-stranded DNA as a consequence of its adenine-like Watson–Crick base pairing with thymine. This unique fluorescence property of 2-AP has been exploited in the design of systems that detect target analytes such as nucleic acids, metal ions and enzyme activities [9–11]. However, the wide spread use of this strategy is limited by the fact that the signal enhancement displayed by 2-AP in nucleic acid-based sensors is often low owing to the existence of stacking interactions with surrounding nucleobases in a single-stranded state, which causes emission quenching.

It is known that incorporation of 2-AP into G-rich sequences results in fluorescence enhancement to a level, which is comparable to that of free 2-AP, owing to formation of a G-quadruplex, a structure formed by stacking complexes comprised of four guanine bases linked via Hoogsteen hydrogen bonding [12–21]. It is assumed that π stacking within the loops of the G-quadruplex is distorted and, thus, that electron transfer quenching of 2-AP by guanine does not effectively occur. As a result, 2-AP in this environment displays strong fluorescence like it does in its free state. Owing to the improved fluorescence properties, 2-AP incorporated in a G-quadruplex has been widely used as a key component in various biosensing platforms, for the detection of various target molecules including metal ions, microRNA, and proteins [19,21,22]. For example, Lee et al. devised a uracil DNA glycosylase (UDG) activity assay, which relies on the formation of 2-AP-incorporated G-quadruplex in response to UDG. Overall, these 2-AP-based approaches overcome the

CONTACT Ki Soo Park kskonkuk@gmail.com, akdong486@konkuk.ac.kr Department of Biological Engineering, College of Engineering, Konkuk University, 120 Neungdong-ro, Gwangin-gu, Seoul, 05029, Republic of Korea

Supplemental data for this article can be accessed here.

limitations of alternative approaches that require expensive and time-consuming modifications of both a fluorophore and quencher [19–21].

The aim of the investigation described below was to explore a simple detection strategy that utilizes a system in which one component of a split G-quadruplex contains an emission silent 2-AP. In addition, the split G-quadruplex components each contain a specific overhang sequence for recognition of the target DNA. In this manner, the G-quadruplex is only formed from the components in the presence of target DNA. Moreover, G-quadruplex formation is associated with a simultaneous enhancement in fluorescence intensity of the 2-AP fluorophore [23]. To maximize the efficiency of a sensor based on this strategy, the effect of the G-quadruplex sequence and split ratios on the fluorescence enhancement of the 2-AP-containing split G-quadruplex was investigated [24]. Finally, to demonstrate its utility in biological applications, the sensor developed in this effort was used for sequence-specific detection of the target DNA, *Chlamydia trachomatis*, one of the most common bacteria that causes a sexually transmitted disease (STD). Importantly, this effort represents the first systematic investigation leading to the development and application of a 2-AP-containing split G-quadruplex-based sensor.

Material and methods

Materials

All DNA samples used in the study were synthesized by Bioneer (Daejeon, Korea) and purified via Bio-RP cartridge purification. The sequences of all DNA samples are listed in Table S1. Tris(hydroxymethyl)aminomethane (Tris), acetic acid (CH_3COOH), potassium acetate (CH_3CO_2K) and magnesium nitrate ($Mg(NO_3)_2$) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade and used without further purification. Ultrapure DNase/RNase-free distilled water (DW) purchased from Bioneer was used in all the experiments.

Sequence-specific DNA detection

Solutions (100 μL), containing 2 μM of S1, 2 μM of S2 (Table S1) and different concentrations of DNA in 10 mM Tris-acetate buffer (pH 7.0) containing 5 mM CH_3CO_2K and 10 mM $Mg(NO_3)_2$, were heated at 90 °C for 5 min, slowly cooled to 25 °C (0.1 °C/s) and incubated at 25 °C for 30 min. Finally, following application of each solution to a well in a black, 96-well plate, its fluorescence intensity was determined with excitation and emission wavelengths of 305 nm and 375 nm, respectively, using a micro-plate reader (Spectramax iD5 Multi-Mode Microplate Reader, Molecular Devices, San Jose, CA). All the experiments were performed in triplicate, and the data were displayed as mean ± SD.

Polyacrylamide gel electrophoresis

The reaction products were resolved on 15% polyacrylamide gel using 1× TBE as a running buffer at a constant voltage of 150 V for 60 min. After staining with GreenStar, the gel image was taken with an UV transilluminator.

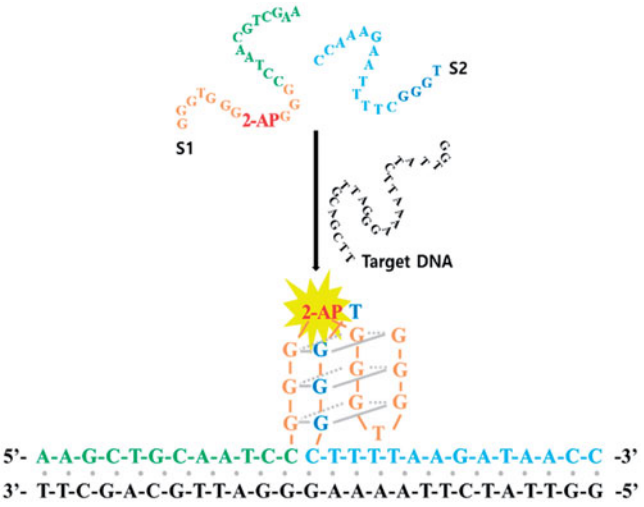

Figure 1. Schematic illustration of the sequence-specific DNA detection using a 2-AP-containing split G-quadruplex (ASG). S1 and S2 are two split G-rich segments linked to a target-specific overhang sequence with S1 only containing 2-AP.

CD measurement

S1, S2 (15 μM) and complementary target DNA (CT; 15 μM) were first added to the reaction buffers (10 mM Tris-acetate, 5 mM CH3CO2K, 10 mM Mg(NO3)2, pH 7.0). The solutions were then heated to 95 °C for 10 min, cooled slowly to 37 °C (0.1 °C/s) and incubated for 30 min, allowing the formation of the G-quadruplex structure, followed by CD measurement at room temperature. CD spectra were recorded on a Jasco-815 spectropolarimeter (Tokyo, Japan) in the range of 230–320 nm in 0.5 mm path-length cuvettes, using a scanning speed of 50 nm/min, a response time of 4 s and a bandwidth of 1 nm.

Sequence-specific DNA detection in human serum

To probe a practical application of the developed sensor, varying concentrations of target DNA were spiked in dilute human serum (1%) and subjected to the same procedure for the sequence-specific DNA detection described above.

Results and discussion

The overall detection procedure

The basic operating principle of the new DNA sensing system, which utilizes a 2-AP-containing G-quadruplex (AG) as the key detection entity, is illustrated in Figure 1. In the sensor, AG is rationally split in a manner that leads to a maximum level of fluorescence enhancement in response to target DNA. Each G-rich segment of the AG is designed to contain a target-specific overhang sequence, in which one contains 2-AP (S1) and the other does not (S2) (Tables S1 and S2). As shown in Figure 1, two split G-rich segments of AG form the active G-quadruplex structure only in the

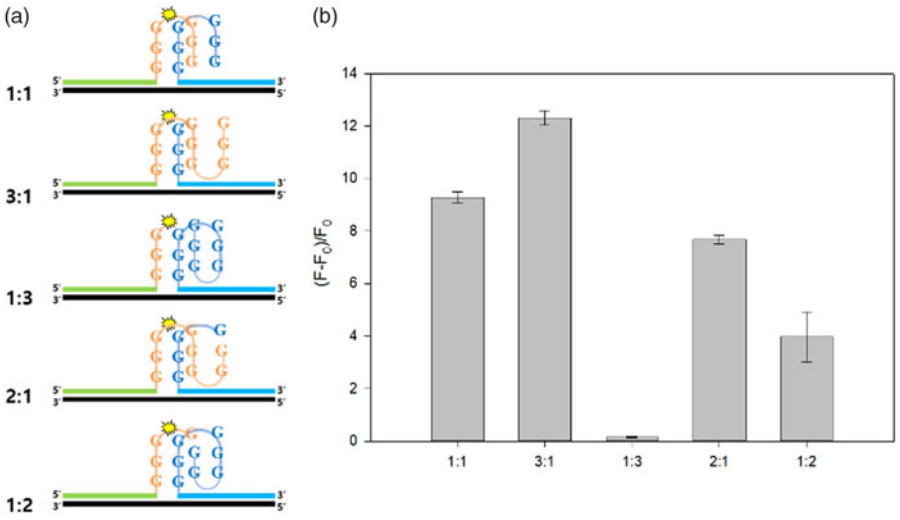

Figure 2. The effect of the G-quadruplex split ratio on the target DNA-induced fluorescence enhancement. (a) Schematic illustration of the 2-AP-containing split G-quadruplex structure with different split ratios (1:1, 3:1, 1:3, 2:1 and 1:2) in the presence of target DNA. (b) The signal-to-background ratio $(F-F_0)/F_0$, where F_0 and F are fluorescence signals in the absence and presence of target DNA, respectively (Figure S2). The final concentrations of S1, S2, and target DNA were 2 μM.

presence of target DNA, a process that blocks stacking interactions of 2-AP with nearby G bases and consequently promotes a dramatic increase in the intensity of fluorescence.

Optimization of G-quadruplex sequence and split ratio

In order to investigate the viability of this strategy and to maximize sensing efficiency, the effect of the G-quadruplex sequence on the target DNA-induced fluorescence enhancement of the 2-AP-containing split G-quadruplex (ASG) was explored. Each of the well-known G-rich sequences, such as PW17 and T30695 (see Table S1 for the sequence information), was split into two segments in a split ratio of 1:1 and then linked to the target-specific overhang sequence [25,26]. As shown in Figure S1, the split G-quadruplex derived from T30695 produced a higher signal enhancement in the presence of target DNA than that coming from PW17. Next, to determine the effect of split ratios, T30695 was divided into five different ratios, including 1:1, 3:1, 1:3, 2:1 and 1:2, based on the number of guanine bases, as illustrated in Figure 2(a). The results of analysis of target DNA using ASGs with the different split ratios show that the signal-to-background ratio $(F–F_0)/F_0$, where F_0 and F represent the fluorescence signal in the absence and presence of target DNA, respectively, was significantly dependent on the split ratio. Specifically, positioning more guanine bases on S1 of the ASG leads to a larger target DNA-promoted fluorescence enhancement. Significantly, a split ratio of 3:1 leads to the largest fluorescence intensity increase in the presence of target DNA, a value that is even larger than that of the frequently used 1:1 split ratio (Figure S2) [16].

Detection feasibility

In the next phase of this study, we demonstrated the feasibility of the new sensor, comprised of the optimized ASG probe with a G-quadruplex derived from T30695 and a 3:1 split ratio, by measuring the fluorescence intensities at 375 nm (F_{375}), an emission maximum of 2-AP (Figure 3(a)). As envisioned, the weak fluorescence signal arising from the sensor in the absence of target DNA (4; Figure 3(a)) significantly increases in the presence of target DNA (5; Figure 3(a)). In addition, target DNAs with different lengths (26, 36 and 46-mer) all effectively promote increases in the fluorescence intensity arising from the probe (Figure S3) [27]. The longer ones generated the slightly, reduced fluorescence signal, which is attributed to the protruding regions that might interfere with the effective formation of active G-quadruplex structure. That these observations are a consequence of target DNA interactions with the probe was evidenced by the results of polyacrylamide gel electrophoresis analysis [28,29]. As shown in Figure 3(b), the split G-rich segments in the presence of target DNA produce new electrophoretic bands, whose positions match those of the hybridization products of target DNA with the split segments (S1 and S2) of ASG. In addition, circular dichroism (CD) spectra were also recorded to examine the conformational changes of probes by the presence of target DNA (Figure S4) [30,22]. Overall, the fluorescence and electrophoretic, and CD results confirm that the target DNA effectively binds to the ASGs and induces formation of active G-quadruplexes with concomitant increases in the fluorescence intensities.

Detection sensitivity and selectivity

The sensitivity of the new sensor was determined next by measuring the fluorescence intensities at 375 nm as a function of target DNA concentration. The results, displayed in the plots in Figure 4, show that the signal-to-background ratio increases with increasing target DNA concentrations up to 2 μM and then slightly decreases at concentrations over 2 μM (Figure 4(a)). The slight reduction is ascribed to binding of the excess target DNAs to only one of the two split segments (S1 and S2; 2 μM), which would result in the non-productive hybridization and consequently reduce the chance to form the active G-quadruplex structure.

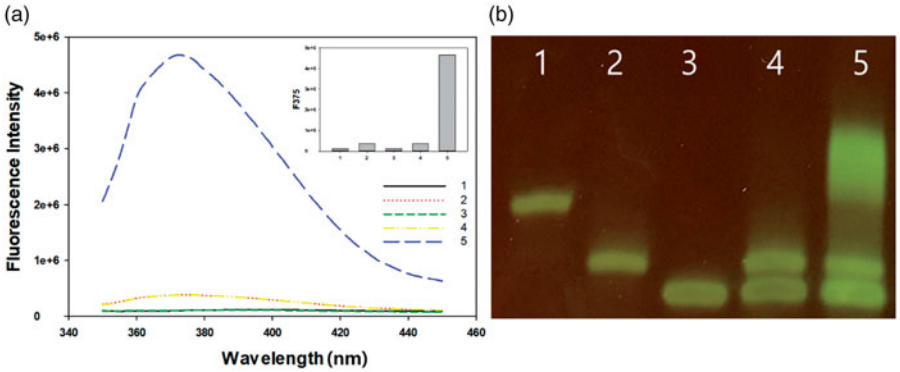

Figure 3. Detection feasibility of the new sensor. (a) Fluorescence intensities and (b) polyacrylamide gel electrophoresis images for samples under different conditions. (1: target DNA; 2: S1; 3: S2; 4: S1 + S2; 5: target DNA + S1 + S2). The final concentrations of S1, S2 and target DNA were 2 μM.

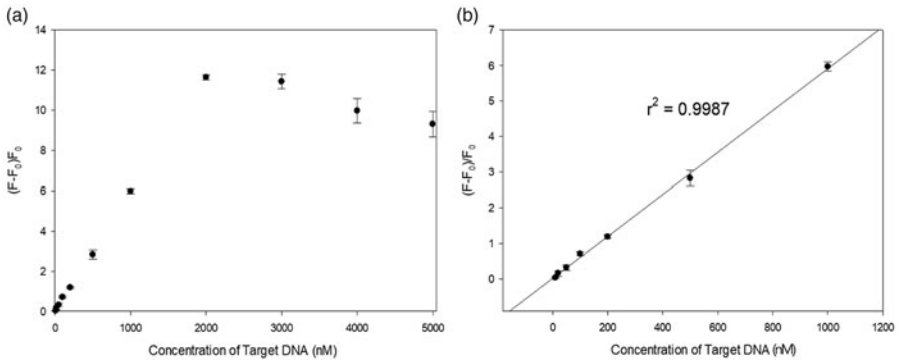

Figure 4. Detection sensitivity of the new sensor. (a) Signal-to-background ratios and (b) linear range of the plot of $(F-F_0)/F_0$ versus target DNA concentration (0–1000 nM). The final concentrations of S1 and S2 were 2 μM.

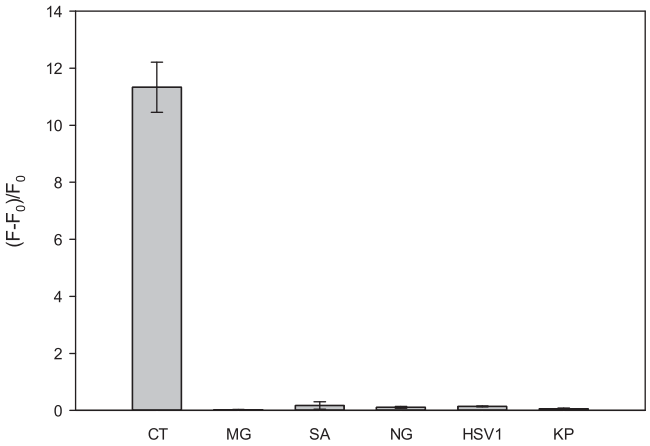

Figure 5. Detection selectivity of the new sensor. The target DNA is *Chlamydia trachomatis* (CT) and not-target DNAs are *Mycoplasma genitalium* (MG), *Staphylococcus aureus* (SA), *Neisseria gonorrhoeae* (NG), Herpes type 1 virus (HSV1) and *Klebsiella pneumonia* (KP). The final concentrations of S1, S2, target DNA and non-target DNAs were 2 μM.

Importantly, an excellent linear relationship ($R^2 = 0.9987$) was found to exist between the level of fluorescence enhancement and target DNA concentration in the range of 0–1000 nM with a limit of detection (3σ/slope) of *ca*. 7.24 nM, which is comparable to those of other DNA detection methods (Figures 4(b) and S5) [11,31–34]. It is important to emphasize that the new sensor system is operationally simple because it is a homogenous assay that utilizes a "mix-and-read" format without the need for separation and/or washing steps [35].

To evaluate the selectivity of the DNA detection system, the abilities of non-target DNAs, the artificially synthesized oligonucleotides which are selected from the whole genome of *Mycoplasma genitalium* (MG), *Staphylococcus aureus* (SA), *Neisseria gonorrhoeae* (NG), Herpes type 1 virus (HSV1) and *Klebsiella pneumonia* (KP) to induce the fluorescence increases were determined and compared with that promoted by target DNA from *Chlamydia trachomatis* (CT). As can be seen by inspection of the graph in Figure 5, a significant fluorescence intensity increase occurs in the presence of the target CT DNA, while the other non-target DNAs promote negligible responses. These results clearly demonstrate that the sensor is highly selective to the target DNA, a finding that lends credence to the proposed working principle of the sensor in which sequence-specific DNA hybridization is critical for the formation of an active G-quadruplex structure.

Practical applicability

Finally, the practical applicability of the new sensor was demonstrated by its use in determining target DNA in human serum [29,36]. It should be noted that circulating, cell-free DNAs in human serum have received great attention as novel biomarkers for non-invasive disease diagnosis [37,38]. To illustrate the practical use of the new sensor, mock clinical samples were prepared by spiking different concentrations of target CT DNA into human serum and then were subjected to the above ASG-based assay procedure. As the results in Figure S6 show, fluorescence intensities of the human serum

Table 1. Determination of target DNA in human serum.

Added (nM)[a]	Measured (nM)[b]	SD[c]	CV (%)[d]	Recovery (%)[e]
50	50.5	0.367	0.726	101
100	111	11.5	10.3	111
250	269	16.4	6.12	107

[a]To measure the concentration of target DNA, a calibration curve was first created by using standards containing known concentrations of target DNA spiked in diluted human serum (1%) (Figure S6). Based on the calibration curve, the F_{375} from the unknown samples was used to determine the concentration of target DNA present in human serum.
[b]Mean of three measurements.
[c]Standard deviation of three measurements.
[d]Coefficient of variation = SD/mean ×100.
[e]Measured value/added value ×100.

samples linearly increase ($R^2 = 0.9937$) with increasing concentrations of the target DNA in the range of 0–1000 nM. Importantly, the target DNA concentrations were determined with excellent precision as evidenced by an observed coefficient of variation (CV) of less than 10.3% and recovery ratios between 101% and 111% (Table 1). This observation confirms the excellent reproducibility and precision of the method and shows its potential for the detection of target DNAs in real clinical samples. In addition, the detection limit was calculated *ca.* 260 nM based on the definition of the limit of detection, 3σ/S, where σ and S are the standard deviation of blank sample and the slope of the linear relationship (Figure S6). Although it is a bit high compared to the one obtained in the pure solution (Figure 4), it is still acceptable to be used in a number of areas such as clinical diagnostics, forensics and biomedicine, after it is combined with PCR that has the exponential amplification capability [39,40].

Conclusion

In the effort described above, we developed a novel, sequence-specific DNA detection method that takes advantage of the unique fluorescence properties of 2-AP incorporated into split G-quadruplex structures. The sensor, containing an optimized sequence and split ratio of the G-quadruplex linked to a target-specific overhang sequence, displays an optimal fluorescence enhancement in the presence of target DNA. Utilizing the optimized sensor, target DNA, the artificially synthesized oligonucleotide which is selected from the whole genome of *Chlamydia trachomatis* was detected with the high selectivity. In addition, the practical applicability of the new sensor was demonstrated by determining the level of *Chlamydia trachomatis* target DNA in human serum. Importantly, the new method is operationally simple and versatile because it not only follows a homogenous assay format and does not require external modification of the fluorophore and/or quencher, but it also is readily amendable to the detection of other target DNAs by proper selection of the target-specific overhang sequence. Owing to these outstanding features, the new sensing system is expected to serve as the foundation for the development of novel biological and environmental sensing platforms.

Disclosure statement

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ORCID

Kyungmoon Park http://orcid.org/0000-0002-0545-0970
Ki Soo Park http://orcid.org/0000-0002-0545-0970

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