Amplified Fluorescent Recognition of G-Quadruplex Folding with a Cationic Conjugated Polymer and DNA Intercalator

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ABSTRACT The single stranded DNA (ssDNA) with G-rich sequence can fold into G-quadruplex via intramolecular hydrogen-bonding interaction in the presence of ligand. This structure conversion can be specifically detected by a fluorescence method based on different interaction between SYBR Green I (SG) and various DNA structures. SG is proved to intercalate into G-quadruplex and results in high fluorescence intensity, which can be further amplified by 6-fold through fluorescence resonance energy transfer (FRET) from a water-soluble cationic conjugated polymer (CCP) to SG due to the high affinity of positively charged CCP to negatively charged rigid G-quadruplex, whereas it is not performed for ssDNA in the absence of K^+. As a result, the ssDNA/SG/CCP complex can be used to detect potassium ions with improved selectivity in a label-free and cost-effective manner.

KEYWORDS: cationic conjugated polymer • fluorescence resonance energy transfer • potassium ion • SYBR Green I

1. INTRODUCTION

Telomeres and telomerase have recently received great attention because of their potential links to cancer, HIV. and other diseases (1−7), and Blackburn et al. won the 2009 Nobel Prize in Physiology or Medicine due to their discovery about telomerases. A unique G-rich DNA sequence in the telomeres was found to protect the chromosomes from recombination, end to end fusion, and degradation through forming G-quadruplexes with highly polymorphic structures in the presence of alkali metal cations (8). This unusual structure and extensive cellular functions make G-quadruplex a very attractive target for drug design, which made it important for determination of G-quadruplex. Various methods such as UV melting curve analysis, circular dichroism, gel electrophoresis, and NMR methods have been applied with high reliability (9−13), which are limited by employing large quantities of DNA and expensive instruments. Some fluorescent dyes (e.g., bisquinolinium and triphenylmethane dyes) are used to recognize G-Quadruplex structure which, however, exhibited relatively low sensitivity and selectivity (14−23). The fluorescence resonance energy transfer (FRET) method with dual-labeled DNA has become a powerful tool to study the structure switch of G-quadruplex with high sensitivity (24). However, the requirement of a dual-labeling process with chromophore and quencher leads to high cost and complicated protocols.

Very recently, a novel FRET strategy employing water-soluble cationic conjugated polymer (CCP) has been developed for improving detection sensitivity and selectivity (25−34). CCPs are a kind of water-soluble macromolecules which possess excellent optical and electronic characters (35−41). The positively charged CCP could bind the negatively charged dsDNA with much stronger electrostatic attraction than single stranded DNA (ssDNA) (26, 28, 29) since dsDNA possessed much higher negative charge density than ssDNA. In addition, the backbone of the CCP molecule consists of a large number of chromophoric repeat units, which enable the high efficient FRET from CCP to the reported chromophore along the whole CCP backbone. For example, the FRET from polyfluorene (P1) to fluorescein (25, 28, 42−44), ethidium bromide (EB) (26, 45), thiazole orange (TO) (27), YOYO-1 (46), and PicoGreen I (PG) (47) results in amplified fluorescence signals and improved sensitivity for detection of DNA and metal ions, etc. Among these methods, the label-free assays using DNA intercalators exhibited high sensitivity and selectivity with low cost and convenient operation (27, 46, 47). For example, SYBR Green I (SG) (48), PG (47), and YOYO-1 (46) had been used for the selective detection of DNA and mercury ions with low cost in a "mix-and-detect" manner. Here, we challenged a label-free method to detect G-quadruplex structure and K^+ with SG and positively charged P1 (Scheme 1). The single-stranded G-rich sequence converts into a more condensed G-quadruplex upon adding K^+ with increasing space charge density. We predicted that SG could interact with G-quadruplex and formed G-quadruplex/SG complex. As demonstrated in Scheme 1, P1 could bind G-quadruplex/SG complex through strong electrostatic interactions, which resulted in the high efficient FRET from P1 to SG and consequently amplified fluorescence signals. With ssDNA/SG/P1 complex

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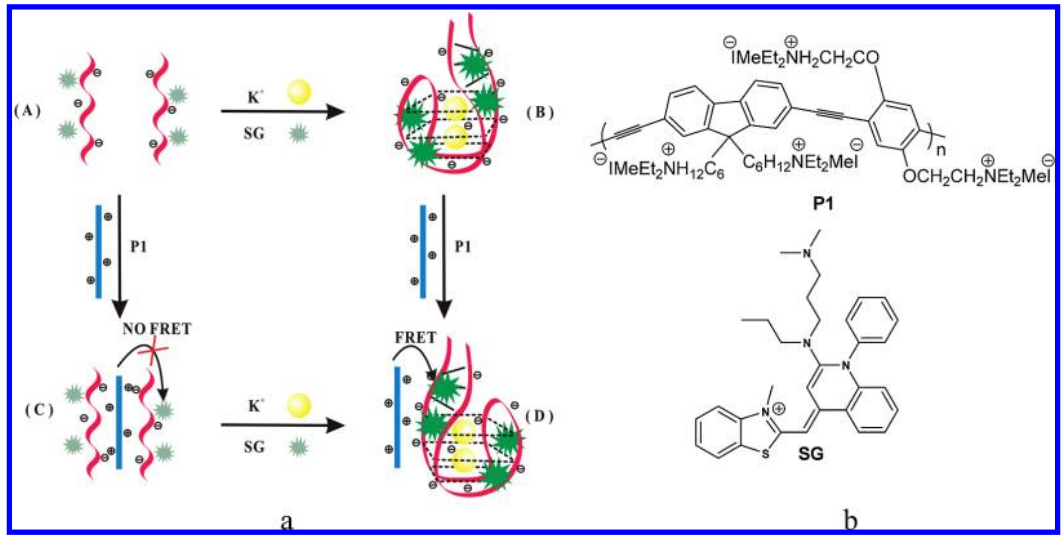
Scheme 1. (a) Schematic Representation of G-Quadruplex Assays with P1 and SG. (b) Chemical Structure of CCP and SG



probe, the DNA structure switch monitoring and quantification analysis of K^+ could be performed with corresponding fluorescence changes.

2. EXPERIMENTAL SECTION

Materials and Instruments. Cationic conjugated polymer P1 was prepared according to a previously published paper (49). DNA oligonucleotides were synthesized and purified by HPLC (Takara Biotech. Co., Dalian). The potassium specific oligonucleotide (PSO, 5′-GGG TTA GGG TTA GGG TTA GGG-3′) and designed sequence of APA-1 (5′-GGG TTA GGG TTA-3′) and APA-2 (5′-TAA GGG ATT GGG-3′) were used to bind K^+ (50, 51). SG (10 000×) was purchased from Invitrogen Inc. A stock solution of 400× was prepared with dimethylsulfoxide (DMSO)/water (volume 1:1) before use. Milli-Q water (18.2 MΩ · cm) was used in all procedures. Tap water was employed in the analysis of real samples. All other chemicals were purchased from China National Pharmaceutical Group Corporation as analytical grade and used as received.

The fluorescence spectra were measured using a Perkin-Elmer LS-55 spectrophotometer equipped with a xenon lamp excitation source. Both the excitation and emission slit width was set to 5.0 nm. SG was excited at 490 nm. Fluorescence resonance energy transfer spectra were measured at an excitation wavelength of 404 nm (for P1). UV−vis absorption spectra were taken on a Shimadzu UV-2550PC recording spectrophotometer.

General Procedures for K^+ Assays. In general, a mixed solution containing 0.8 μM PSO or APA (including APA-1 and APA-2) and 50 mM KCl was incubated for 30 min at 25 °C, Milli-Q water was used instead of KCl as the blank. Five microliters of SG (400×) was added and incubated for another 5 min at RT. An equal concentration of KCl solution was used to make up 2 mL for fluorescence detection (excited at 490 nm). For further amplification experiments, 10^{-7} M P1 (final concentration) was then added into an APA/SG/KCl mixture and the fluorescence spectra were recorded (excited at 404 nm) by a LS-55 spectrometer. For real sample analysis, tap water was used instead of Milli Q water. The sensor's sensitivity was investigated by analyzing the fluorescence change of P1/APA/SG complex upon addition of different concentrations of KCl (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mM). For selectivity analysis, 50 mM of various kinds of salts (NaCl, NH_4Cl, LiCl, $CaCl_2$, or $MgCl_2$) were used instead of KCl.

Procedures to Optimize SG Intercalation and P1 Amplification. The intercalation kinetics of SG with G-quadruplex was studied as follows: 0.8 μM APA-1 and APA-2 and 50 mM KCl were mixed and incubated for 30 min at 25 °C. After addition of SG, the solution was incubated for different times (from 1 to 10 min), and the fluorescence spectra were measured.

The concentrations of SG and P1 were investigated in this work. The mixture containing 0.8 μM APA-1, 0.8 μM APA-2, and 50 mM KCl was incubated for 30 min at 25 °C; 5 μL SG of different concentrations was added and incubated for another 5 min at RT. An equal concentration of KCl solution was used to make up 2 mL for fluorescence detection, and the final concentration of SG was 2.45×10^{-7}, 4.9×10^{-7}, 9.8×10^{-7}, 1.96×10^{-6}, and 2.45×10^{-6} M, respectively. Then, P1 was added in a 10^{-7} M final concentration, and the fluorescence spectra were measured. For experiments aimed to optimize the P1 concentration, 5 μL of SG (400×) was used and different concentrations of P1 were investigated (2.5×10^{-8}, 5×10^{-8}, 1×10^{-7}, and 1.5×10^{-7} M in final concentration).

3. RESULTS AND DISCUSSION

Sensor Operation Principle: Assay for G-Quadruplex Folding of DNA. SG is used as a sensitive reagent for staining dsDNA in DNA qualification and quantification; for example, SG has been widely applied in gel electrophoresis and real-time PCR (52). The strong binding of SG with dsDNA by minor groove binding led to ~11 times higher fluorescence than ssDNA, which implies SG could discriminate ssDNA and dsDNA structures (53). We predicted that the positively charged SG could bind to highly negatively charged G-quadruplex with higher affinity than ssDNA through the strong electronic attraction and π−π stacking of conjugated molecules. We first challenged SG discrimination of a DNA structure change from PSO, a 21 mer potassium specific sequence, that has been proved to form G-quadruplex in the presence of K^+ as reported in our and others' studies (13, 24, 45). As demonstrated in Figure S1 (Supporting Information), the fluorescence of SG increased by 5-fold after addition of K^+, which implied the formation of G-quadruplex and SG intercalation into the G-plane. In addition, the designed sequence of APA-1 and

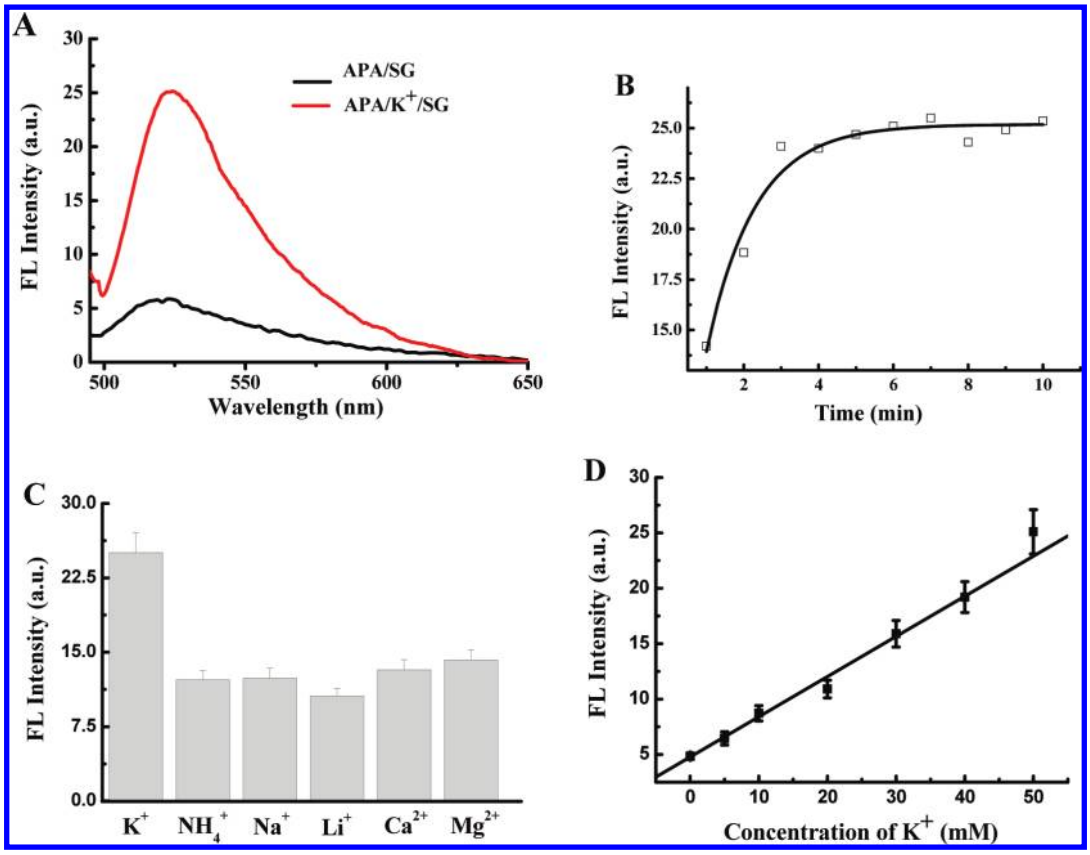


FIGURE 1. (A) Fluorescence spectra of APA/SG in the absence (black line) or presence (red line) of K^+. (B) Fluorescence intensity of APA/SG/KCl at different time points after addition of SG. (C) Emission intensity at 525 nm of APA/SG in the presence of 50 mM various metal ions and (D) different concentrations of K^+. [APA1] = [APA2] = 1.0×10^{-8} M, [SG] = 1.96×10^{-6} M. [KCl] = 50 mM (for A, B, and C).

APA-2, cut from PSO in appropriate position without disturbing binding activity with K^+, exhibited rapid binding kinetics and low intramolecular secondary structure. Those two oligos could recombine into integrity G-quadruplex structures in the presence of K^+ (50, 54, 55). As illustrated in Figure 1A, the single stranded APA did not bind SG and resulted in low fluorescence in the absence of K^+. Upon addition of 50 mM K^+, APA folded into G-quadruplex structure and resulted in improved fluorescence intensity by 5-fold, which implied that the two oligos successfully recombined into G-quadruplex and SG intercalated the G-planes. Thus, the APA was chosen for investigating the DNA structure change and consequently the K^+ assay. The kinetic of SG intercalation with G-quadruplex was studied, and we found that the fluorescence intensity increased to the highest signal within 5 min (Figure 1B). The selective and rapid intercalation of SG into G-quadruplex structure formed the basis of a convenient method for detection of the G-quadruplex and following quantification analysis of K^+. As shown in Figure 1C, the fluorescence intensity of APA/SG induced by K^+ was about twice of that induced by an equal concentration of other metal ions (including Na^+, NH_4^+, Li^+, Ca^{2+}, and Mg^{2+}), with linear relation for K^+ ranging from 5 to 50 mM (Figure 1D). Unfortunately, the low sensitivity and poor selectivity of APA/SG probe limited their application, and a further amplification procedure was required to improve the sensor's performance.

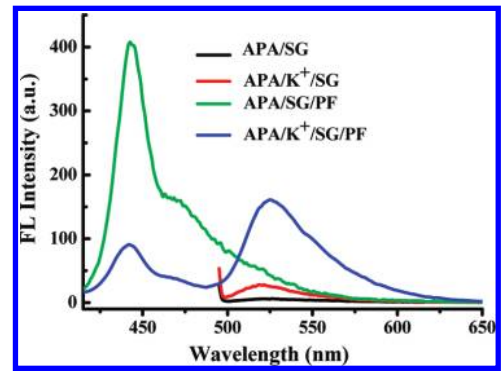


FIGURE 2. Fluorescence spectra of APA/SG in the absence (black line) or presence (green line) of P1; fluorescence spectra of APA/SG/KCl in the absence (red line) and presence of PF (blue line).

Cationic conjugated polymers (CCPs) possess excellent optical characters and are usually employed to amplify fluorescence signals due to their unique "molecular wire" characters. CCPs with twisted conjugated backbone and highest positive charge density have been proved to facilitate their electrostatic attraction to rigid DNA structures (such as dsDNA); for example, P1 has been testified as an excellent fluorescence probe to differentiate ssDNA and dsDNA with improved sensitivity (56). Here, P1 was used in this work to amplify the fluorescence of SG/G-quadruplex complexes through FRET, and the absorption spectrum of SG overlaps well with the emission spectra of the P1 which enabled efficient energy transfer between CCPs and the DNA intercalators (Figure S2, Supporting Information). As demon-

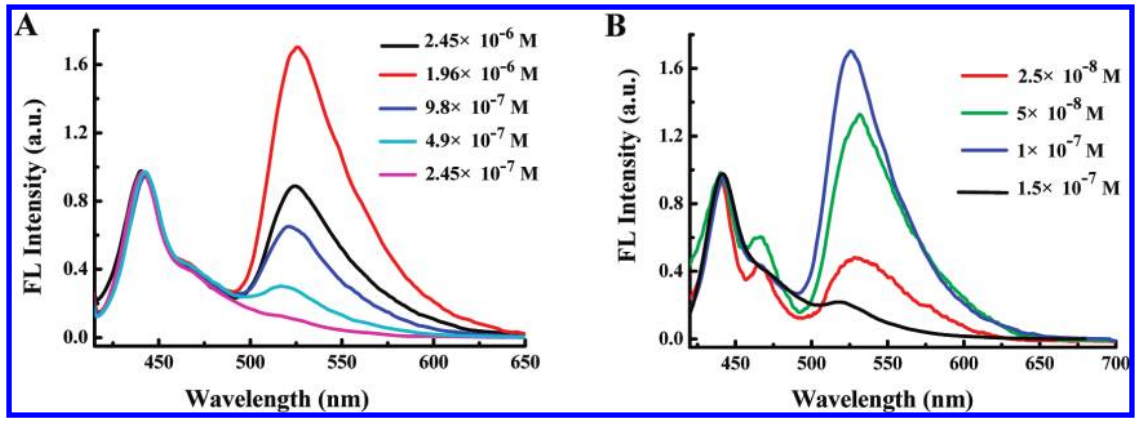


FIGURE 3. Optimization of SG intercalation and P1 amplification. Normalized emission spectra of P1/APA/SG/KCl with different concentration of SG (A) and P1 (B). [APA-1] = [APA-2] = 1.0×10^{-8} M, [K^+] = 50 mM, the concentration of SG was from 2.45×10^{-7} M to 2.45×10^{-6} M while P1 was 1.0×10^{-7} M; the concentration of P1 was from 2.5×10^{-8} M to 1.5×10^{-7} M while SG was 1.96×10^{-6} M.

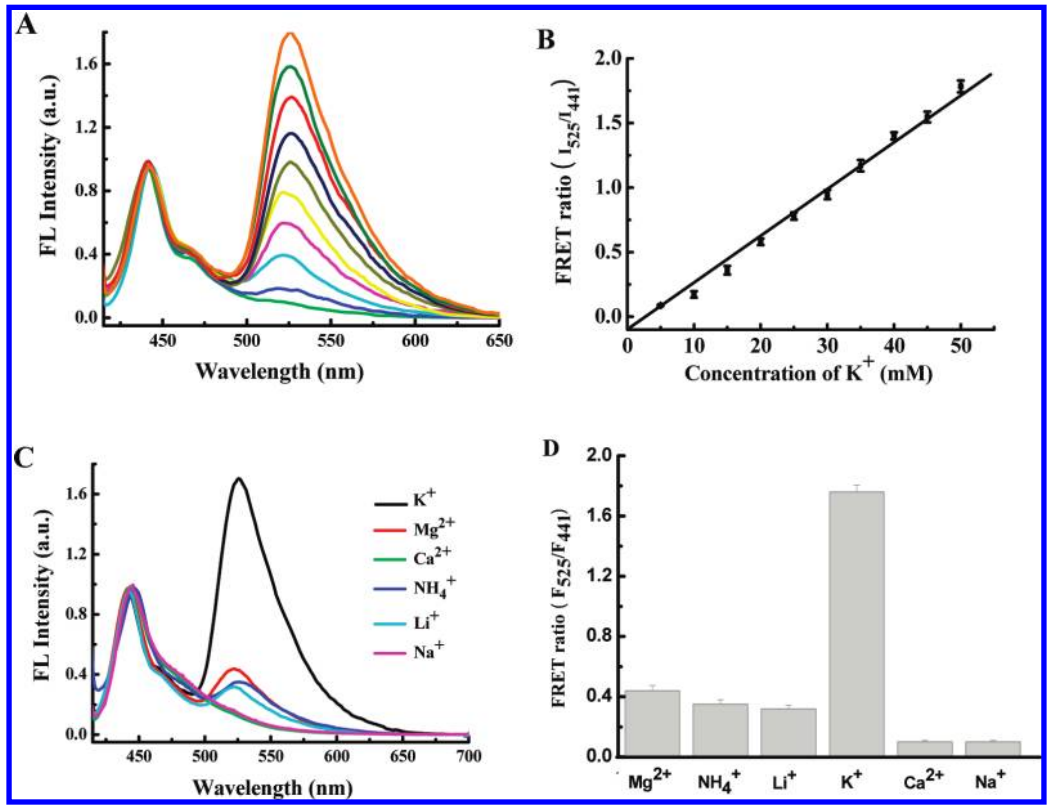


FIGURE 4. Sensitivity and selectivity analysis. Normalized emission spectra (A) and FRET ratio (F_{525}/F_{441}) (B) of P1/APA/SG in the presence of different concentrations of K^+ (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mM); Normalized emission spectra (C) and FRET ratio (I_{525}/I_{441}) (D) of P1/APA/SG in the presence of 50 mM metal ions. [APA-1] = [APA-2] = 1.0×10^{-8} M, [SG] = 1.96×10^{-6} M, and [P1] = 1.0×10^{-7} M.

strated in Scheme 1, APA could selectively bind with K^+ and formed G-quadruplex structures. SG bound with G-quadruplex and resulted in high fluorescence. The addition of P1 resulted in the high efficient FRET from P1 to SG and amplified fluorescence signals because P1 could bind G-quadruplex/SG complex through strong electrostatic interactions. As shown in Figure 2, the fluorescence at 532 nm was 25 for G-quadruplex/SG, which was enhanced by 6-fold through FRET from P1 to SG after the addition of P1, whereas FRET was not performed for the single stranded APA due to the weak affinity between ssDNA with P1, and no significant peak at 532 nm was found (42). In general, the key force between P1 and phosphate groups in DNA is electrostatic attractions which brings highly negative charged G-quadruplex to P1 much closer than ssDNA. In addition, the positively charged P1 acts as competitive DNA binding agents to repel free SG from ssDNA and, thus, resulted in low fluorescence intensity (26). Briefly, the strong interaction of SG and G-quadruplex together with the tight binding of P1 with G-quadruplex made P1 and SG closer and then resulted in high efficient FRET.

Optimization of P1 Binding and SG Intercalation. The concentrations of SG and P1 were optimized to improve sensitivity and selectivity. As demonstrated in Figure 3A, keeping DNA at 1.0×10^{-8} M and KCl at 50 mM,

1.96×10^{-6} M of SG led to the highest fluorescence intensity. In addition, 1.0×10^{-7} M P1 was suitable for providing the highest FRET efficiency from P1 to SG (Figure 3B). In the following experiments, the sequences of APA-1/APA-2 (each 1.0×10^{-8} M), SG (1.96×10^{-6} M), and P1 (1.0×10^{-7} M) were employed to quantitatively detect K^+. For all following FRET-related results, the spectra are normalized with respect to the emission of P1.

Sensitivity and Selectivity Analysis of K^+. Figure 4A shows that, under the optimized conditions, addition of KCl causes a concomitant increase in the emission intensities of SG and FRET efficiency. The FRET ratio of the intensity at 525 nm to that at 441 nm (I_{525}/I_{441}) was used to analyze FRET from P1 to SG. The FRET ratio increased along with the increase of KCl concentration (Figure 4B), which implied the random coil structure of APA converted to the G-quadruplex. The FRET ratio was dependent on the concentration of K^+ with a linear relationship ($R = 0.995$) over a wide range of 5−50 mM. We also challenge this method for K^+ assay in real samples, in which several parallel tap water samples were employed (Table S1, Supporting Information). The results described that the K^+ assay could be performed in real samples, and low concentrations of impurity in drinking water did not interfere with the performance.

The specificity of this method was investigated by use of other metal ions such as Na^+, Li^+, NH_4^+, Ca^{2+}, and Mg^{2+} instead of K^+. Figure 4C,D compared the emission spectra and FRET ratio (F_{525}/F_{441}) of the P1/APA/SG in the presence of the various metal ions, where F_{525} and F_{441} were the fluorescence intensity at 525 and 441 nm, respectively. The FRET is very weak in the presence of Na^+ and Ca^{2+}, while a small increase in fluorescence of SG is observed in the presence of Mg^{2+}, NH_4^+, and Li^+. The FRET ratio (F_{525}/F_{441}) for K^+ is approximately 16 times higher than those for Na^+ and Ca^{2+} and 4 times higher than those for Mg^{2+}, NH_4^+, and Li^+ (Figure 4D). We can achieve high selectivity for K^+ over Na^+, Li^+, NH_4^+, Ca^{2+}, and Mg^{2+} using P1/APA/SG complex as probe in homogeneous solution. In contrast to results using APA/SG probe in the absence of P1, whose fluorescence induced by K^+ is only twice of that by other ions (Figure 1C), the selectivity by employing P1 as amplification factor was significantly improved. This phenomenon can be explained by the different binding mode between SG and different DNA structures. SG binds G-quadruplex with much higher affinity than ssDNA through electrostatic and intercalate interaction. Besides that, the positively charged P1 will compete with SG in binding to DNA, and some SG molecules will be repelled away from DNA, i.e., the addition of P1 leads to the dissociation of the SG molecules from ssDNA and results in nonfluorescence. Therefore, the nonspecific binding between SG and ssDNA in the absence of K^+ was greatly decreased, and the selectivity of this method was improved.

4. CONCLUSION

In this paper, we reported a label-free fluorescence method for discrimination of G-quadruplex structure and ssDNA and K^+ detection using CCP and DNA intercalator (SG). SG could discriminate G-quadruplex and ssDNA because the intercalating of SG into G-quadruplex resulted in improved fluorescence signals. This discrimination could be further amplified by employing P1, and the fluorescence improved by 6-fold through FRET using P1 as a light harvesting complex. This label-free and low-cost method could detect K^+ in a wide linear range from 5 to 50 mM with excellent selectivity, and various kinds of metal ions (e.g., Na^+, NH_4^+, Li^+, Mg^{2+}, and Ca^{2+}) could not result in increased fluorescence of SG. In addition, this method might be extended to detect other secondary structures by investigating their interaction with SG.

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Supporting Information Available: UV−vis and FL spectra of SG and P1, experiments with PSO, and detection of K^+ in real samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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