An Aptamer-based and Pyrene-labeled Fluorescent Biosensor for Homogeneous Detection of Potassium Ions

Cuiping MA,[†] Heqing HUANG, and Chunhui ZHAO

Key Laboratory of Eco-chemical Engineering, Ministry of Education, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, P. R. China

A simple and homogeneous method based on aptamer and pyrene moieties for the detection of K⁺ was developed. The aptamer was labeled by pyrene moiety at 3' end as the molecular recognition element. In the presence of K⁺, the complementary oligonucleotide labeled by pyrene moiety at 5' end was displaced from the aptamer, which was accompanied by a dramatic decrease of the excimer fluorescence of pyrene. However, the excimer fluorescence remains in the absence of the target. With optimum conditions, relative changes of the pyrene excimer fluorescence intensity were proportional to the concentrations of K⁺ in the range of 6.3×10^{-4} to 1.0×10^{-2} M with a detection limit of 5.0×10^{-4} M. Importantly, in the presence of Na⁺, NH₄⁺, Mg²⁺ and Ca²⁺ cations of biological fluids, this method was able to detect K⁺ with high selectivity. In a word, the assay seems to have great potential applications, especially in biological fluids due to its simplicity, specificity and homogeneous detection.

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Introduction

Potassium ions (K⁺) play a significant role as a transmitter in cells and in the regulation of membrane potential, nerve stimulation, and hormone secretion.¹ Abnormal K⁺ concentrations usually lead to several diseases. So specific recognition and quantitative detection of K⁺ are extremely crucial in fundamental research as well as in clinical practice. However, most of the reported studies concerning K⁺ avoided their clinical applications. For example, Minta *et al.*² and Crossley *et al.*³ had reported K⁺ detection in biological fluids that were based on fluorophores PBFI and coumarin diacid cryptand [2.2.2], respectively. However, poor selectivity against Na⁺ was shown because they only tolerated 1.5- and 3.4-fold excesses of Na⁺ in biological fluids, respectively. The development of practical polydiacetylene (PDA) liposome-based micro arrays to selectively detect potassium ions in the presence of Na⁺ had been reported by Lee *et al.*⁴ The detection limit for the 30 min of incubation results in the microscope images was 0.5 mM. Other K⁺ detection methods were also reported based on FRET (fluorescence resonance energy transfer) and aptamers modified by fluorophores.⁵⁻⁷ In 2005, Nagatoishi *et al.* reported thrombin-binding aptamer labeled by pyrene moieties at both ends for the detection of potassium in the presence of an excess of Na⁺.⁸ However, these methods were greatly limited in their applicability because modified aptamers may cause reduction of affinity and specificity.⁹⁻¹² Recently, Shi *et al.* reported an aptamer-based optical biosensor for K⁺ detection, in which aptamer was unmodified as the molecular recognition element.¹³ It is very difficult and expensive to label oligonucleotides with pyrene at both ends and such methods are unsuitable for

extensive applications. Therefore, we used two pyrene-labeled oligonucleotides for K⁺ detection in this paper to circumvent the above described disadvantages. In addition, a visual method for K⁺ detection was recently developed by Yang *et al.*,¹⁴ which made K⁺ detection simpler than before.

Pyrene is a well-characterized excimer-forming fluorophore which has been incorporated into synthetic oligonucleotides by several research groups.¹⁵⁻¹⁹ The fluorescence wavelength of pyrene monomer is about 395 nm. The fluorescence wavelength is switched to about 485 nm, when two pyrene molecules are brought into close proximity to form an excimer.^{20,21} The excimer fluorescence with characteristically large Stokes shift can be easily discriminated from the monomer fluorescence, thereby allowing homogeneous hybridization assays to be done even in the presence of excess unhybridized probes.²² Therefore, the experiment labeled by pyrene moiety overcomes common disadvantages, such as being tedious and time-consuming, which exist in the majority of hybridization experiments that use oligonucleotide probes artificially labeled with radioisotopes, fluorescent dye molecules, enzymes and biotin.²³ Moreover, the pyrene excimer has a longer fluorescent lifetime (40 ns) than that of the most of biological environment species (<5 ns).^{24,25} These two special features of pyrene excimer will make it have great potential application in biological environments.

In this report, we describe a simple and homogeneous assay method, which is based on aptamer and excimer-forming fluorophore pyrene. The aptamer and its complementary oligonucleotide were each labeled with one pyrene moiety, one in the 3'-position and the other in the 5'-position. Upon hybridization, the two fluorophore moieties came into close proximity and formed an excimer. In this paper, the thrombin-binding aptamer was chosen for K⁺ detection. Aptamer forms a chair-type quadruplex structure upon binding to K⁺.²⁶⁻²⁸ Therefore, in the presence of K⁺, the complementary oligonucleotide labeled by pyrene moiety at 5' end was displaced

[†] To whom correspondence should be addressed.
E-mail: sc169@163.com

Fig. 1 General strategy for aptamer-based and pyrene-labeled fluorescent sensor and chemical structures of the pyrene labeled oligonucleotide.

from the aptamer. Simultaneously, a dramatic decrease of the excimer fluorescence could be observed.

Experimental

Materials

All oligonucleotides were synthesized by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. and purified by HPLC. The thrombin-binding aptamer was the sequence d (GGT TGG TGT GGT TGG) for K^+ detection, which was modified by pyrene moiety at 3' end. The complementary oligonucleotide was the sequence d (CCA ACC ACA CCA), which was modified by pyrene moiety at 5' end. The sequence in boldface was complementary with the italic portion in the aptamer sequence. All chemicals were analytical grade and were used without further treatment. All solutions were prepared in deionized water.

Apparatus and preferences

All analyses were performed with a Hitachi F-4500 spectrophotometer (Tokyo, Japan) equipped with a xenon lamp. Fluorescence spectra were measured at an excitation wavelength of 340 nm and an emission wavelength at 485 nm. The slit width was 10 nm. The oligonucleotide quantitation was carried out on a Cary 50 UV-VIS-NIR spectrophotometer (Varian).

General procedure

The spectroscopic measurement experiments were carried out in 5 mM Tris-HCl (pH 7.2) containing 20 mM NaCl and 2 mM $MgCl_2$. A 1 M K^+ stock solution was prepared by dissolving proper amounts of KCl in the buffer in a 1-mL volume and diluting it 10 fold twice to 10 mM. All solutions were prepared by appropriate dilution from this standard. Similarly, the other stock solutions of cations were prepared by dissolving appropriate amounts of different NaCl, NH_4Cl , $MgCl_2$ and $CaCl_2$ in the buffer described above. The fluorescence spectra of solutions were recorded at an excitation wavelength of 340 nm and an emission wavelength of 485 nm.

Experiment of quantitative analysis

Aptamers and the complementary oligonucleotide were diluted from 1.0×10^{-5} M stock solution to 0.4 μ M in the buffer, and incubated at room temperature for 15 min to form an excimer. With the addition of K^+ , the reaction solution was incubated for 5 min at room temperature for binding of K^+ with aptamer, and the excimer fluorescence decrease was observed. The assay procedures for sodium, ammonium, calcium, and

Fig. 2 Fluorescence changes of different concentrations of K^+ ions (0, 0.1, 0.6, 1, 2, 4, 6, 10 mM) with 0.4 μ M heteroduplex of aptamer and complementary oligonucleotide. Buffer: 5 mM Tris-HCl, 20 mM NaCl, 2 mM $MgCl_2$, pH 7.2. λ_{ex} = 340 nm.

magnesium ions were the same as that for the K^+ , except for using NaCl, NH_4Cl , $CaCl_2$, and $MgCl_2$ stock solutions. All the experiments were repeated three times.

Results and Discussion

Preparation of the complementary oligonucleotide probe

The relative stability of heteroduplex was predicted by the mfold program (available at the web site, <http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi>) to help select the appropriate complementary sequence to form an excimer.^{29,30} On the basis of thermodynamic prediction and K^+ reaction buffer, a 12-bp complementary oligonucleotide modified by pyrene moiety at 5' end was designed. The complementary oligonucleotide was fully hybridized with the aptamer.

The fluorescence measurements to detect target K^+ ions

The principle of aptamer-based and pyrene-labeled fluorescent biosensor for K^+ is shown in Fig. 1. In the presence of K^+ , the decrease of pyrene excimer fluorescence was observed for complementary oligonucleotides displaced from aptamers by targets. Therefore, an excimer-emission intensity decrease depending on the concentration of K^+ could be exhibited. To prove the sensitivity of the method, we tested the detection of different concentrations of K^+ by fluorometric titration with KCl (Fig. 2). Figure 2 showed the excimer fluorescence intensity decrease upon the addition of K^+ . In the absence of K^+ , the fluorescence spectrum exhibited no change of the excimer fluorescence intensity with two small monomer peaks at 380 and 395 nm. It was reported that the fluorescence of the pyrene monomer coexisted when two pyrene moieties were labeled at the same end of the oligonucleotide.¹⁷ With the addition of K^+ , the resulting excimer-emission intensity decreased, owing to releasing the free complementary oligonucleotides, which brought a slight but steady increase in the emission peaks at 380 and 395 nm.

The sensitivity and specificity for detecting target K^+ ions

According to the protocols described above, the detection and quantification of K^+ ions had been achieved. The correlation curve of relative changes in excimer fluorescence intensity was

Fig. 3 Plots of the relative changes in fluorescence intensity (%) of heteroduplex of aptamer and complementary oligonucleotide with different concentrations of KCl (■), NaCl (●), MgCl₂ (◆), CaCl₂ (▲), and NH₄Cl (▼). $\lambda_{\text{ex}} = 340$ nm. [Heteroduplex] = 4.0×10^{-7} M.

constructed against the concentrations of K⁺ ions (Fig. S1 (Supporting Information)). Therefore, quantitative analysis of an unknown K⁺ ions concentration was also achievable. With the addition of K⁺ ions, the decrease in excimer emission was observed in the concentration range of 0–100 mM. At the same time, a linear dependence up to 10 mM K⁺ ions was achieved. The detection limit for the 5 min of incubation was 0.5 mM, which allowed good results because the concentration of K⁺ ion in serum is in the range of 3.5–5.3 mM. The methodology showed excellent sensitivity for the detection of K⁺ ions.

To assess the specificity of the method, we also monitored changes in excimer fluorescence of oligonucleotide probes in the presence of Na⁺, Mg²⁺, Ca²⁺, and NH₄⁺ ions. The relative changes of excimer fluorescence intensities were plotted against the amount of K⁺ ions (Fig. 3). With the addition of different cations, only the excimer emission of K⁺ decreased dramatically. For Na⁺, Ca²⁺, Mg²⁺ and NH₄⁺ ions, the changes of relative excimer-emission intensities were very weak due to their weak binding affinity with thrombin-binding aptamer.^{8,31} Hence, the proposed method represents good specificity for K⁺ ions over Na⁺, Mg²⁺, Ca²⁺, and NH₄⁺ ions at experimental condition.

K⁺ ions coexist with an excess of Na⁺ ions in extracellular conditions. At present, the specificity for K⁺ against Na⁺ ions was rather low for many studies concerning K⁺ ions sensors, so such the monitoring of K⁺ in extracellular condition was very difficult.^{32,33} Thereby the study of the interference of Na⁺ becomes very essential. In this paper, the dependence of the excimer emission was examined in the presence of Na⁺ and K⁺ ions for the interference of Na⁺ (Fig. S2 (Supporting Information)). Fortunately, 2 mM KCl could still be well detected in the presence of 100 mM NaCl. The interference result from Na⁺ further indicated our method seemed to be very good selectivity for K⁺ ions against Na⁺ ions.

Conclusions

A simple and homogeneous assay had been developed for the detection of K⁺ ions, and the method can be exploited for the monitoring any targets such as proteins, DNA and RNA by the forming and change of the fluorescence of pyrene excimer

emission. The excimer fluorescence could be easily discriminated from the monomer fluorescence, thereby allowing homogeneous hybridization assays to be done even in the presence of excess unhybridized probes. Therefore, the methodology presented here represents an excellent sensitivity for K⁺ detection. The method demonstrated a linear relationship with the concentration of K⁺ ions ranging from 6.3×10^{-4} to 1.0×10^{-2} M, and a detection limit of 5.0×10^{-4} M was achieved. Especially, the interference from Na⁺ ions was very minor. The good selectivity for K⁺ against other species such as Na⁺ allows our method great potential applications in biological environment.

In addition, the simultaneous emission of the excimer and monomer forms of pyrene, and the presence of an isobestic point in the fluorescence spectrum shown on Fig. 2 present the interesting possibility of using this sensor as a ratiometric probe. More importantly, the pyrene excimer has a much longer fluorescence lifetime (40 ns) than that of the biological background (5 ns), and time-resolved measurements can be used to eliminate the biological background.^{23,34} Therefore, it is very possible to detect K⁺ ions in serum and living cells by time-resolved fluorescence measurements by our method with its simplicity, sensitivity, specificity and homogeneous detection.

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Supporting Information

Additional information as noted in text. This material is available free of charge on the web at <http://www.jsac.or.jp/analsci/>.

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