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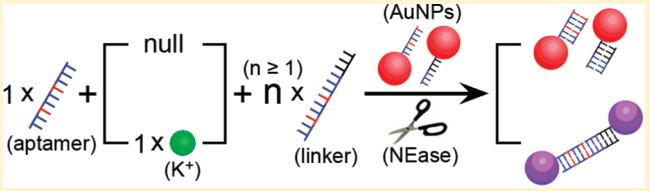
Fabrication of a Highly Sensitive Aptasensor for Potassium with a Nicking Endonuclease-Assisted Signal Amplification Strategy

Xiaoli Zhu,[†] Jing Zhao,[‡] Yao Wu,[†] Zhongming Shen,[†] and Genxi Li*[,†,‡]

[†]Laboratory of Biosensing Technology, School of Life Sciences, Shanghai University, Shanghai 200444, P. R. China

[‡]Department of Biochemistry and National Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, P. R. China

ABSTRACT: A novel strategy to fabricate an aptasensor for potassium with high sensitivity and selectivity by using nicking endonuclease is proposed in this work. A nicking endonuclease (Nt.CviPII), which may recognize specific nucleotide sequences in double-stranded DNA formed by a potassium-binding aptamer and a linker DNA but cleave only the linker strand, may transfer and amplify the quantitative information of the potassium detection to that of the linker DNA through elaborate strand-scission cycles. Since the technique for gene assay is much more mature, the linker DNA can thereby be detected by a number of available methods. Here, taking advantage of a simple and fast gold nanoparticles-based sensing technique, we are able to assay the linker and consequently potassium ion simply by UV−vis spectroanalysis and even with the naked eye. Results show that a 2 μL sample containing 0.1 mM of potassium is enough to induce distinct color appearance of the nanoparticles, and the potassium ion can be easily distinguished from many other ions. The strategy proposed in this work shows some unique advantages over some traditional methods and may be further developed for the detection of some other chemicals in the future.



Aptamers are artificial DNA/RNA oligonucleotides that are selected in vitro by systematic evolution of ligands by exponential enrichment (SELEX).[1] They can bind to various targets, from small molecules to whole cells, thus have been considered as a unique substitute for antibody in the field of biosensors and therapeusis since the first description in 1990.[2−6] In addition to their wide-range recognition ability, aptamers also have advantages over antibodies since they can be not only engineered completely in a test tube but also readily produced by chemical synthesis. Besides, they may possess desirable storage properties and have little or no immunogenicity in therapeutic applications. Therefore, in the last few years, aptamers have been broadly used in the fabrication of biosensors for the detection of ions,[7−9] small biological molecules,[10−12] proteins,[13−15] and even whole living cells,[16−18] bringing about a new branch of biosensors as aptasensors.

Despite the success in the development of aptasensors, most of the reported architectures suffer from low sensitivity, which is mainly caused by the lack of amplification of the detection signal. Like the well-known enzyme-linked immunosorbent assay (ELISA), enzyme-linked amplification is also a possible solution for the fabrication of sensitive aptasensors.[19,20] However, because of the difference in the properties between nucleic acids and proteins, such as electrical characteristic, flexibility, etc., unpredictable problems may occur. For example, the combination of aptamer with protein enzyme may result in the uncorrected folding of the oligonucleotides, which is adverse to the binding of aptamer with its target.

As is well-known, the particularity of nucleic acids may also provide possible amplification for detection signal, one of which is the well-known polymerase chain reaction (PCR), while rational application of nuclease is another favorable approach. Though both of the possible solutions derived from the system of nucleic acids have been widely used in the amplified assay of genes,[21−25] to the best of our knowledge, only a few amplification strategies by using PCR or nuclease is reported to develop sensitive aptasensors.

In this work, taking advantage of a special kind of restriction endonucleases, nicking endonuclease (NEase), we have proposed a nicking endonuclease-based aptasensor (NEBAS) for potassium (K^+). In this strategy, the NEase recognizes specific nucleotide sequence in double-stranded DNA but cleaves only one of the two strands. With the elaborate design of the DNA, PCR-like strand-scission cycles are achieved, which may result in n ($n \geq 1$) times of cleaved oligonucleotides. Moreover, we have introduced gold nanoparticles (AuNPs) to convert the abundant oligonucleotides product to amplified distance-depended surface plasmon absorption signal. It allows us to detect the K^+ simply and sensitively by UV−vis spectroanalysis and even with the naked eye.

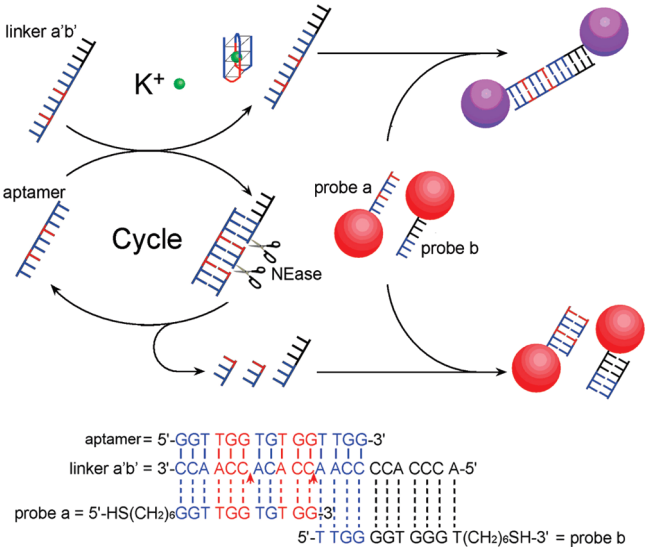
EXPERIMENTAL SECTION

Chemicals. The DNA oligonucleotides (Guaranteed Oligos, HPLC-purified) were synthesized by Invitrogen. Detailed sequences are shown in Scheme 1. NEase (Nt.CviPII) was from New England Biolabs. Chloroauric acid ($HAuCl_4$), tris(2-carboxyethyl)phosphine (TCEP), and other chemicals were all of

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Scheme 1. Schematic Description of the NEBAS



analytical grade and used as obtained. All solutions were prepared with doubly distilled water, which was purified with a Milli-Q purification system (Branstead) to a specific resistance of >18 MΩ cm.

Preparation of Functionalized Gold Nanoparticles. AuNPs with an average diameter of 13 nm were synthesized by a citrate reduction method,[26,27] and the AuNPs-DNA conjugate were prepared following the published protocol with minor modifications.[28] In brief, a sodium citrate solution (1%, 3.5 mL) was rapidly added to a boiled $HAuCl_4$ solution (0.01%, 100 mL) under vigorous stirring. The mixed solution was boiled for 10 min and further stirred for 15 min. The resulting wine-red colloidal AuNPs were cooled to room temperature and filtered through a 0.22 μm membrane filter. The colloid was then concentrated 4-fold by centrifugation at 12 000 rpm for 20 min. The concentration of the as-prepared AuNPs was estimated to be 14 nM, which was calculated from the quantity of starting material ($HAuCl_4$) and the size of the nanoparticles.

The as-prepared AuNPs were divided into two sets to be separately covalently functionalized with probe a and b oligonucleotides. The thiol-modified probe DNA was first activated by TCEP in a TA buffer (20 mM Tris-acetate, pH 7.9, the concentrations of probe DNA and TCEP were 1 and 10 mM, respectively) for 1 h and then incubated with AuNPs (final concentrations of probe DNA and AuNPs were 3 μM and 14 nM, respectively) for 20 min. The mixture was "aged" in salt and brought to a final concentration of 0.1 M NaCl through a stepwise process. After another incubation of 16 h, the mixture was centrifuged at 12 000 rpm for 20 min to remove the excess reagents. The precipitate was washed by a TA buffer (20 mM Tris-acetate, pH 7.9) and recentrifuged twice. Finally, the functionalized AuNPs were redispersed in TA buffer (20 mM Tris-acetate, pH 7.9) to a concentration of 14 nM and were ready for use.

Nicking Endonuclease-Based Amplification. The detection target K^+ was first allowed to form a complex with a potassium-binding aptamer and then join into NEase-based strand-scission cycles, which may transfer and amplify the quantitative information of the K^+ detection to that of a linker DNA. All the solutions used here were prepared in a TA buffer (20 mM Tris-acetate, pH 7.9), which has been tested to be suitable for both the folding of aptamer and the exertion of NEase. The detailed procedure was as follows: (1) An aqueous solution of KCl sample (2 μL, the concentration of K^+ was in a range of 0−20 mM) was mixed with potassium-binding aptamer (2 μL, 2 μM) and incubated together for 40 min at 37 °C to form aptamer-K^+ complex. Zn^{2+}, Cu^{2+}, Mg^{2+}, Na^+, and NH_4^+ (100 mM) were adopted as controls. (2) Linker a′b′ (4 μL, 10 μM or 100 μM, aptamer/linker a′b′ = 1:10 or 1:100) was further added and incubated with the aptamer-K^+ complex for 10 min at 37 °C to hybridize with the unoccupied aptamer. (3) The sample was brought to a final volume of 40 μL by adding a NEase-containing working buffer (0.2 units in 32 μL of TA buffer). The NEase-based strand-scission reaction was sustained for 60 min at 37 °C and terminated by a 65 °C water-bath for 20 min. Also, the test solution was ready for further assay after cooling down to room temperature.

Gold Nanoparticle-Based Colorimetric Assay. To obtain the quantitative information of K^+, the above test solution was added to probe DNA functionalized AuNPs directly (test solution/probe a functionalized AuNPs/probe b functionalized AuNPs = 2:1:1 in volume). The surface plasma absorption of AuNPs was collected by a Libra S22 UV−vis spectrophotometer (Biochrom, England), and the color was recorded by a Canon PowerShot A650 digital camera. All the experiments were conducted at least four times, and the data points presented were averages from at least four independent experiments.

Polyacrylamide Gel Electrophoretic Analysis. Besides the AuNPs-based colorimetric assay, the samples of oligonucleotides mixture were also monitored for comparison by denaturing 10% polyacrylamide gel electrophoresis. The gel was stained with SYBR Green II.

RESULTS

The NEBAS for K^+ with amplified detection strategy has been shown in Scheme 1. The potassium-binding aptamer is complementary to a linker (linker a′b′) which is seven bases longer than the aptamer at the 5′-end. The double stranded DNA (dsDNA) formed by the aptamer and the linker has two restriction sites (5′-(cut)CCD-3′, D = G or A, marked in red, Scheme 1) for NEase (Nt.CviPII), while the enzyme cleaves only the linker strand to three fractions. Because of the low melting temperature (Tm), these fractions disassociate from the aptamer, thus the aptamer is free to hybridize with another intact linker, forming a new substrate for NEase to cleave and produce more fractions. As a result, strand-scission cycles are launched without the need for a thermal cycling procedure and a large amount of linker DNA molecules can be cleaved into fractions in the presence of only a little of aptamer. Therefore, after probe DNA functionalized AuNPs (probe a functionalized AuNPs/probe b functionalized AuNPs = 1:1) are added in the test solution, although the linker is complementary to the probes modified on the surface of the nanoparticles, the cleaved species cannot combine the nanoparticles together via hybridization, keeping the color of AuNPs unchanged. Nevertheless, in the presence of K^+, the potassium-binding aptamer prefers to bind with the ions to form aptamer-K^+ complex rather than to hybridize with the linker. The shortage of dsDNA consequently results in the silence of NEase and the accumulation of a large molar excess of the intact linker, which further induces the aggregation of the probe DNA functionalized AuNPs, presenting a red-to-purple color change. In a brief summary of the strategy, the quantitative information of K^+ is transferred and amplified to that of the linker DNA, which

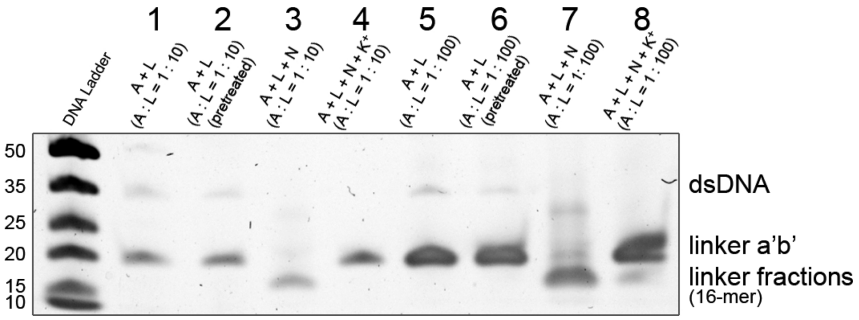


Figure 1. Polyacrylamide gel electrophoresis analysis: lane 1, the aptamer and the linker are mixed without pretreatment; lane 2, the oligonucleotides are pretreated by heating to 90 °C, cooled down to 37 °C slowly to unfold the DNA chains before being mixed; lane 3, not pretreated but in the presence of NEase (0.2 units); lane 4, not pretreated but in the presence of both the target K^+ (20 mM) and the enzyme NEase (0.2 units). Lanes 1−4 shows the bands obtained with the ratio of aptamer/linker 1:10, while lanes 5−8 1:100 ratio. The "A", "L", and "N" are abbreviations of aptamer, linker a′b′, and NEase, respectively.

is subsequently detected by AuNPs-based colorimetric sensing. As a result, an amplified detection of K^+ is achieved.

In order to confirm that our strategy can indeed work well, polyacrylamide gel electrophoretic analysis (PAGE) has been first employed to check the behavior of the oligonucleotides during the assay roughly but straightforward. Lanes 1, 2, 5, and 6 in Figure 1 show the bands of the dsDNA formed by the aptamer and the linker (aptamer/linker = 1:10 for lanes 1 and 2 and 1:100 for lanes 5 and 6). In the cases of lanes 1 and 5, the oligonucleotides are mixed directly, while for lanes 2 and 6, the oligonucleotides are pretreated by heating to 90 °C and cooling down to 37 °C slowly to unfold the DNA chains before being mixed. As is shown by the figure, in all the cases, a band for dsDNA and another band for the excess linker can be observed and no significant difference can be observed between lanes 1 and 2 or between lanes 5 and 6, suggesting that hybridization may also occur under no pretreatment. It is reasonable since the sequences of the aptamer and the linker do not allow self-folding. So the procedure to unfold the DNA chains can be neglected, and we may simplify the following experiments by using the DNA chains without any pretreatment.

Lanes 3 and 7 in Figure 1 show the bands of aptamer and linker a′b′ in the presence of NEase (aptamer/linker = 1:10 for lanes 3 and 1:100 for lanes 7). It can be observed that only a band with a fast migration rate is obtained, which is ascribed to the linker fractions cleaved by the NEase. The results suggest that in both cases, strand-scission cycles are launched (10 cycles for lane 3 and 100 cycles for lane 7), in which 10-fold or even 100-fold of the linker can be cleaved completely. Nevertheless, if the aptamer has been incubated with K^+ before it is mixed with 10-fold of the linker, it can be known from lane 4 in Figure 1 that there is no band corresponding to the linker fractions, suggesting that the strand-scission cycles are totally blocked. Furthermore, as is shown in lane 8, even if 100-fold of the linker is present, only a small quantity of the linker can be cleaved. These results mean that the aptamer-K^+ complex may block the exertion of NEase, and a relatively small quantity of the complex will result in a large quantity of uncleaved linker. Therefore, the quantitative information of K^+ can be successfully transferred and amplified by several dozen times to the quantitative information of the linker DNA (as is shown in lane 8 of Figure 1). The next step to sensitively detect the target is to convert the amplified information to detectable signals.

We fix the ratio of aptamer to linker a′b′ at 1:10 to obtain completely suppressed strand-scission cycles in the

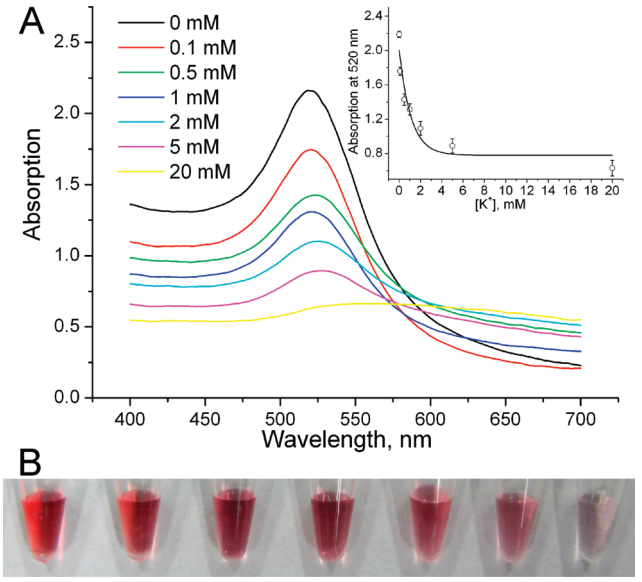


Figure 2. The (A) absorption and (B) color appearance of the AuNPs mixed with the test solution containing different amounts of the target detection K^+. From left to right in part B, the concentration of K^+ is 0, 0.1, 0.5, 1, 2, 5, and 20 mM, respectively. The insert in part A shows the relationship between the absorption peak of AuNPs at 520 nm and the concentration of K^+.

AuNPs-based sensing process. Figure 2 shows the absorption and color of the probe DNA functionalized AuNPs, which have been added with the NEase-treated oligonucleotides test solution (a solution containing the aptamer, linker, NEase, and/or K^+). As is expected, without K^+, the linker is cleaved by NEase to fractions, so it cannot combine the dispersed AuNPs together, keeping the wine-red color of AuNPs stable. Nevertheless, if K^+ is present, the linker cannot be cleaved and, consequently, may hybridize with the oligonucleotides on the surfaces of the nanoparticles. As a result, the intact linker-induced aggregation of AuNPs is triggered in varying degrees depending on the concentration of K^+ in the test solution, presenting red-to-purple colors as appearance (Figure 2B). Furthermore, the higher the concentration of K^+ is, the more drastic the color and the absorption of AuNPs changes. Thereby, the color or absorption of AuNPs can be used as detectable signals for the assay of K^+, and 0.1 mM K^+ can be enough to show a distinct color appearance.

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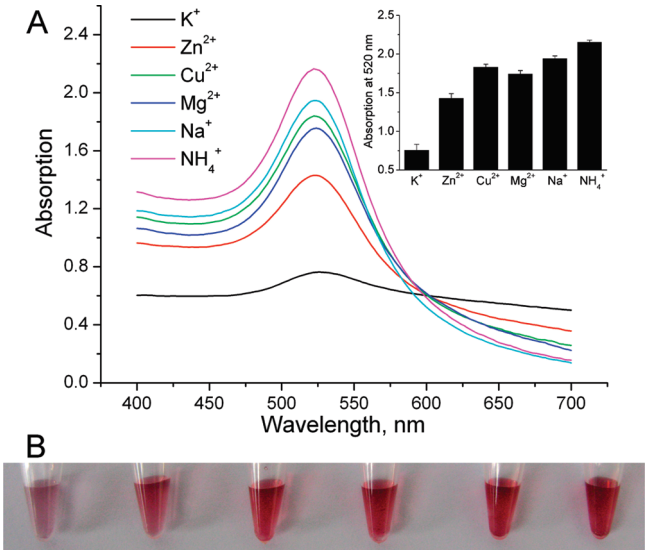


Figure 3. The (A) absorption and (B) color appearance of the AuNPs mixed with the test solution containing different kinds of detection ions. From left to right in part B, the ions are K^+ (10 mM), Zn^{2+} (100 mM), Cu^{2+} (100 mM), Mg^{2+} (100 mM), Na^+ (100 mM), and NH_4^+ (100 mM), respectively. The insert column figure in part A shows the absorption peak of AuNPs at 520 nm in the cases of different ions.

Meanwhile, the absorption value at the peak (520 nm) also has a negative correlation with the concentration of K^+, which can be fitted by half of a sigmoidal curve (Figure 2A, insert).

We have further examined the selectivity of the NEBAS. Some ions such as Na^+, NH_4^+, Zn^{2+}, Mg^{2+}, and Cu^{2+} are employed for the control experiments. As is shown in Figure 3, AuNPs may keep the color of red in the presence of any of these control ions, even though their concentration is 10 times higher than that of K^+. The remarkable difference of the color between the target K^+ and the control ions suggest that the proposed NEBAS in this work is of considerable selectivity. It is reasonable, since only the target ion K^+ is able to form a complex with the potassium-binding aptamer.

DISCUSSION

The K^+ detection sensitivity with the proposed NEBAS in this work is ~0.1 mM, while only as high as ~250 mM K^+ can give distinct color appearance in a previously reported colorimetric approaches, in which the covalent AuNPs-probe DNA strategy was also employed.[29] Obviously, the improvement in this work is attributed to the application of NEase, which not only favors the signal amplification but also makes the detection of the target transferred from K^+ to easier-detected DNA. With optimization of the unit, acting time of NEase, and the ratio of the aptamer to the linker, the sensitivity may be further improved.

Recently, Lu et al. reported the comparison of two types of AuNPs-based sensing strategies.[30] The conclusion is that the covalent AuNPs-probe DNA strategy, which we adopt here in this work, is simpler to use and more versatile but relatively time-consuming, while another noncovalent AuNPs-probe DNA strategy has better sensitivity, shorter operation time, but lower antijamming ability. We have demonstrated in this work that the NEase-assisted covalent AuNPs-probe DNA strategy may also has considerable sensitivity, which may rival the noncovalent strategy.[31,32] So, with integration of the advantages of the two types of strategies, the NEase-assisted AuNPs-based sensing strategies may be more competitive in the development of aptasensors.

We should also mention the strategy to fabricate the aptasensor in this work. In a conventional aptasensor, the quantitative information of target is usually converted to detectable signals directly. Taking fluorescence resonance energy transfer (FRET)-based aptasensors for instance, the target-induced conformational changes of fluorophore/quencher labeled aptamer may draw the fluorophore and quencher together or apart, producing detectable FRET signals. However, since the target may vary from small ions to huge cells and the regular conformational changes of the corresponding aptamers are usually unknown, it is difficult to make an architecture of aptasensor universal for the detection of different targets. Nevertheless, with a transfer of the quantitative information of the detection target to that of a linker DNA through NEase, for example, the aptasensor can be transferred to a "DNA sensor", in which a number of universal sensing strategies are available. The concept though requires an additional procedure may create a platform for the design of universal aptasensors that can be used for the detection of other molecules. For instance, it has been known that another NEase Nt.AlwI has a restriction site 5′-GGATCNNNN(cut)N-3′, which overlaps with the complementary sequence of the aptamer of platelet-derived growth factor (PDGF) exactly.[33] So, on the basis of the strategy presented in this paper, PDGF should be also sensitively detected by employing Nt.AlwI-based strand-scission cycles.

CONCLUSIONS

In summary, we have proposed a novel NEBAS for K^+ with high sensitivity. The strategy is based on the fact that a NEase may recognize a specific site in the dsDNA formed by the potassium-binding aptamer and a linker DNA but cleave only the linker. Automatic strand-scission cycles can thereby be launched at room temperature, since the aptamer will dissociate from the cleaved linker and combine with another intact linker. However, when K^+ is present, the cycles are interrupted, resulting in the accumulation of uncleaved linker. There is a positive relationship between the uncleaved linker and K^+. So, with the benefit of the strand-scission cycles, a novel kind of NEBAS for K^+ with high sensitivity and selectivity is proposed. We have also discussed the advantage and prospect of the NEBAS.

AUTHOR INFORMATION

Corresponding Author

*E-mail: genxili@nju.edu.cn.

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