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Split Aptamer Mediated Endonuclease Amplification for Small-Molecule Detection

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A novel, highly sensitive split aptamer mediated endonuclease amplification strategy for the construction of aptameric sensors is reported.

Aptamers are in vitro selected artificial single-stranded nucleic acids that bind a specific target with high selectivity and affinity.\(^1\) Compared to conventional recognition elements such as antibodies, aptamers possess advantages including low cost, synthetic convenience, chemical stability, modification flexibility.\(^2\) Hence, they have been widely used for constructing biosensors and shown their enormous potential in detecting various targets.\(^3\)-\(^8\) A typical strategy for aptamer sensor is conformational change or structure switch in which the secondary structures of aptamers undergo a change on interacting with their targets.\(^9,10\) An alternative design is to utilize the sandwiched format in which two aptamer probes are used for recognition of the target and the formation of triplex complex is responsible for the detection.\(^11\) In this direction, we and others have reported a proximity-dependent hybridization strategy for protein detection by using two aptamers binding to different sites of the target.\(^12\)-\(^14\) Alternatively, a strategy called split aptamer has been developed by using two aptamer fragments, and this strategy has been adopted for detecting various targets.\(^15\)-\(^18\) However, current split aptamer assays have been rarely coupled to signal amplification methods, leading to limited sensitivity of these assays. The challenge for combining signal amplification approaches with split aptamer lies in the lacking of downstream biochemical reactions that are specific to the assembly of split aptamer fragments.

Herein, we develop a novel, highly sensitive split aptamer mediated endonuclease amplification (SAMEA) strategy for the construction of aptameric sensors. To our knowledge, this is the first time that split aptamer has been coupled to a signal amplification method. Therefore, it may provide a new paradigm for the design of ultrasensitive aptameric sensors, because of incorporation efficient signal amplification in split aptamer can substantially enhance the detection sensitivity. To demonstrate the concept, we choose two small molecules, adenosine and cocaine, as the model targets in this study. The results revealed that the developed strategy offers ~10^5-fold sensitivity improvement as compared to previous amplification-free split aptamer assays.\(^19\)-\(^21\)

Endo IV is a DNA repairing enzyme that cleaves phosphodiester bonds of the apurinic or apyrimidinic (AP) sites within DNA double strands.\(^22,23\) This selectivity for double-strand DNA allows efficient signal amplification and detection for DNA or RNA targets with linear structures.\(^24\) We realize that the assembly of split aptamer fragments in response to its target actually brings two tail sequences in close proximity, forming a three-way junction structure that is essential for proximity-dependent hybridization.\(^12\) This realization motivates us to investigate the hypothesis of using the three-way junction structure as the substrate for DNA endonuclease IV (Endo IV). Our results reveal that Endo IV cleaves efficiently the substrate in a three-way junction structure with substantial signal amplification (see ESI for details: Fig. S1-S3, Table S1). Based on this new finding, a new split aptamer strategy with signal amplification is then developed via a combination of split aptamer with proximity-dependent hybridization mediated Endo IV amplification, as illustrated in Scheme 1. This design employs the Endo IV-mediated amplification for detecting the assembly of the two split aptamer fragments. Two aptamer fragment probes (AFP) are designed by splitting the whole aptamer sequence at a proper site followed by introducing short stabilizer sequences at two ends to help the complementation of two fragments. Two tail sequences are further extended at the terminals of the aptamer fragments for the formation of a three-way junction structure (Scheme 1 A and Table S1, ESI). In the presence of target, the two split aptamer fragments are assembled into a stable complex on interacting with its target. This assembly draws the two tail sequences into close proximity and enables them to hybridize stably with the detection probe, forming a three-way junction. The detection probe has an apurinic or apyrimidinic (AP) site for Endo IV cleavage flanked by two sequences complementary to the two tail sequences of AFP1 and AFP2, respectively. It delivers a low fluorescent background in its intact state because of efficient Förster resonance energy transfer (FRET) between its two labels, the fluorophore FITC and the quencher TAMRA. Once the three-way junction structure is formed, the detection probe is cleaved by Endo IV and its fragments are too short to stably hybridize with the tail sequences. Hence, the tail sequences in the duplex of AFP 1 and AFP 2 are released and allowed to hybridize with another detection probe, mediating a cycling of the cleavage of the detection probe. This cycling is able to activate an intense fluorescence signal because of the separation of
the fluorophore FITC from the quencher TAMRA and thus creates substantial signal amplification for detecting target concentration. In contrast, when target is absent, these two aptamer fragments are not able to form a stable complex because the stabilizer sequences are too short to hybridize each other at the reaction temperature. In this case, the proximity-dependent hybridization of the two tail sequences with the detection probe fails and no cleavage of the detection probe occurs, thereby retaining a low fluorescent background.

![Scheme 1](https://example.com/scheme1.png)

Scheme 1. Design of split aptamer mediated endonuclease amplification (SAMEA).

Figure 1 depicts typical fluorescence spectral responses of the SAMEA strategy for adenosine detection. Incubation with one of the two aptamer fragments and the detection probe in the presence of Endo IV, we only obtained a very weak fluorescent peak at 518 nm, implying efficient FRET based quenching of FITC by TAMRA and little cleavage of the detection probe by Endo IV. This was ascribed to the very low annealing temperatures, ~13.5 °C and ~17.5 °C, respectively, for hybridization between two aptamer fragments and the detection probe. Incubation of both aptamer fragments with the detection probe in the presence of Endo IV gave a slight increase of the fluorescent peak at 518 nm. This suggested that two aptamer fragments were assembled together to some degree to mediate low-efficiency cleavage for the detection probe. In contrast, incubation of two aptamer fragments, the detection probe and Endo IV in the presence of adenosine displayed a strong fluorescent peak at 518 nm with a signal-to-background ratio of ~5.8. This manifested efficient cleavage of the detection probe by Endo IV, indicating the assembly of two aptamer fragments on interacting with target adenosine and implying the potential of the developed strategy for adenosine detection. A control experiment using inosine, a deaminated analogue of adenosine, in place of adenosine also did not show an increased fluorescent peak, which testified the assembly of aptamer fragments was specific to target adenosine. In addition, we found that the fluorescence spectra, though displaying substantial variations for the peak at 518 nm, the intensities at 580 nm for the emission of TAMRA almost remained unchanged. This finding suggested the possibility of using a ratiometric method for the detection of adenosine, which might increase the robustness of the assay.

**Figure 1.** Typical fluorescence spectral responses of the SAMEA for adenosine detection. Adenosine AFP1 + detection probe + Endo IV (red), adenosine AFP2 + detection probe + Endo IV (green), adenosine AFP1 + adenosine AFP2 + detection probe + Endo IV (cyan), inosine + adenosine AFP1 + adenosine AFP2 + detection probe + Endo IV (blue), adenosine+adosine AFP1 + adenosine AFP2 + detection probe + Endo IV (pink). Reactions were performed at 37 °C for 2.5 h and detection probe 1 μM, adenosine AFP1 100 nM, adenosine AFP2 100 nM, adenosine 5 mM, inosine 5 mM, Endo IV 1U were used for all experiments.

Agarose gel electrophoresis analysis was performed to verify the formation of the aptamer fragments’ assembly and the proximity-dependent cleavage reaction. As shown in Figure 2, the image gave a clear band for the detection probe and aptamer fragment 1, while yielded two bands for aptamer fragment 1, which was attributed to the formation of a dimer of this fragment. The mixture of two aptamer fragments with adenosine showed a new band of ~30 bp, which was ascribed to the formation of the aptamer fragment assembly. Surprisingly, we did not observe a new band for the mixture of two aptamer fragments, the detection probe and adenosine. This result suggested that the detection probe did not form a stable complex with the aptamer fragment assembly, presumably due to the fact that the detection probe was too short to hybridize stably with the aptamer fragment assembly. Interestingly, after incubation with Endo IV with this mixture, the band for the detection probe disappeared with the appearance of a new band for a short sequence. This band for the short sequence was ascribed to the cleaved product of the detection probe, evidencing effective cleavage of the detection probe mediated by the assembly of aptamer fragments. This phenomenon revealed an interesting property that Endo IV-mediated cleavage of AP site seemed not to require a stable complex for the DNA duplex. In contrast, we still obtained a clear band for the detection probe in the mixture of two aptamer fragments and the detection probe and Endo IV in the absence adenosine. This result confirmed that cleavage of the detection probe was specific for the adenosine-induced assembly of aptamer fragments, verifying the specificity of the SAMEA strategy.

**Figure 2.** Agarose gel electrophoresis images of detection probe and adenosine split aptamer fragments with Endo IV. Lane 1, DNA marker (10–300 bp); lane 2, 2 μM detection probe; lane 3, 2 μM adenosine AFP1; lane 4, 2 μM adenosine AFP2; lane 5, 5 mM adenosine + 1 μM adenosine AFP1 + 1 μM adenosine AFP2; lane 6, 5 mM adenosine + 1 μM adenosine AFP1 + 1 μM adenosine AFP2 + 1 μM detection probe; lane 7, 5 mM adenosine + 1 μM adenosine AFP1 + 1 μM adenosine AFP2 + 1 μM detection probe + 1 U Endo IV; lane 8, 1 μM adenosine AFP1 + 1 μM adenosine AFP2 + 1 μM detection probe + 1 U Endo IV.
The SAMEA strategy was found to be very specific for the detection of target adenosine (Fig. S4, ESI). The detection of four other nucleotides using the SAMEA strategy merely gave marginal changes in the fluorescence response at 518 nm. This result revealed that the proposed strategy has an excellent selectivity for adenosine against other nucleotides.

The performance of the developed strategy for quantitative analysis of adenosine was further investigated, as shown in Figure 3. Increasing concentrations of adenosine from 5 pM to 5 mM were found to result in gradual increases in the fluorescence peak at 518 nm. A plot of the fluorescence intensities versus the logarithmic adenosine concentration showed a linear correlation in the five-decade range from 5 pM to 50 nM. The calibration equation was \( f = 3.9531 + 1.4268 \log c \), with a correlation coefficient \( R^2 = 0.9816 \), where \( f \) is the fluorescence intensity and \( c \) is the concentration of adenosine. The detection limit was estimated to be 3 pM according to 3\( \sigma \) rule. This results revealed that the developed strategy offers \( >2 \times 10^2 \)-fold sensitivity improvement as compared to previous amplification-free split aptamer assays.\(^{19,21}\) Such a low detection limit and a wide dynamic range were also much better than existing fluorescent aptamer based strategies for adenosine detection assays,\(^{25-27}\) revealing that the SAMEA strategy realized a highly sensitive platform for adenosine assay.

To demonstrate its generality, the developed strategy was further applied to cocaine assay. Typical fluorescence spectral responses of the SAMEA strategy for cocaine detection were also performed (Fig. S7, ESI). Incubation of aptamer fragments with the detection probe in the presence of Endo IV gave a weak fluorescent peak at 518 nm. This result confirmed that two aptamer fragments were not able to be assembled together efficiently so as to mediate the cleavage of the detection probe. Incubation of two aptamer fragments, the detection probe and Endo IV in the presence of cocaine displayed a strong fluorescent peak at 518 nm with a signal-to-background ratio of \( \sim 4.1 \). This observation manifested efficient cleavage of the detection probe by Endo IV because of the assembly of aptamer fragments on interacting with cocaine, implying the potential of the developed strategy for cocaine detection. This assembly of aptamer fragments was very specific to cocaine. Actually, we found that benzylecgonine, a main metabolite of cocaine, did not show appreciable increase in the fluorescent peak. In addition, weak fluorescence responses were also obtained for other species possibly coexisting with cocaine in buffer solution (Fig. S8, ESI), testifying the high selectivity of the developed strategy for cocaine assay.

![Fluorescence spectra of SAMEA assay in response to adenosine of various concentrations.](image)

**Figure 3.** (A) Fluorescence spectra of SAMEA assay in response to adenosine of various concentrations. (B) The corresponding fluorescence response at 518 nm of the SAMEA assay. Inset: linear relationship between fluorescence peak intensity and the logarithm of adenosine concentration. Error bars are standard deviation of three repetitive experiments.

Besides the end-point fluorescence detection in the SAMEA strategy for quantifying the target, it is also possible to use real-time monitoring of the activated fluorescence signal for quantification (Fig. S5, ESI). Interestingly, we observed quasi-linear increases for the fluorescence intensities at 518 nm for different target concentrations. These data suggested that the Endo IV-mediated amplification followed a quasi-linear kinetics. Such a quasi-linear kinetics was ascribed to a much lower concentration of target-induced assembly of split aptamer as compared to the Michaelis constant.\(^{28}\) Plotting of the fluorescence activation rates also revealed a dynamic correlation with the target concentrations in the range from 5 pM to 5 mM with a linear correlation to the logarithmic adenosine concentration from 5 pM to 50 nM. A benefit with the real-time fluorescence monitoring was that the assay time could be reduced substantially, say, measuring the time-dependent fluorescence changes for 10 min and calculating the fluorescence activation rates for quantification.

To evaluate the SAMEA assay in complex biological media, the detection of adenosine in 10% human serum samples was also performed. Five sera samples with added adenosine of different concentrations were measured and satisfactory recoveries between 90.4% and 108.4% were obtained (Fig. S6 and Table S2, ESI), indicating the potential of the SAMEA strategy for real sample analysis.

Notes and references

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