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An ultrasensitive fluorescent aptasensor for adenosine detection based on exonuclease III assisted signal amplification

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ABSTRACT

We report here a graphene oxide (GO)-based fluorescent aptasensor for adenosine detection by employing exonuclease III (Exo III) as a signal amplifying element. In the absence of adenosine, the adenosine aptamers hybridized with the complementary DNA (cDNA), and the Exo III could not cleave the single-strand signal probes labeled with carboxylfluorescein (FAM) at its 5′ ends. When the graphene oxide was finally added, it could strongly adsorb the single-strand signal probes and quenched the fluorophore effectively. In the presence of adenosine, the aptamers associated with the targets, which led to the formation of duplex DNAs between the cDNAs and the signal probes. The Exo III thereafter could digest the duplex DNAs from 3′ blunt terminus of signal probes, liberating the fluorophore. Upon adding the GO, the fluorophore could not be adsorbed and quenched. By coupling cyclic enzymatic cleavage, a remarkable fluorescent increase was obtained. Due to the specific recognition ability of the aptamer for the target and the powerful quenching property of GO for signal probe, this proposed approach has a good selectivity and high sensitivity for adenosine. In the optimum conditions described, >100% signal enhancement was achieved and a limit of detection as low as 1 nM was obtained, which is lower than those of commonly used fluorescent aptamer sensors. Moreover, the biosensor exhibited an ultrahigh sensitivity and held a versatile platform for clinical diagnostics, molecular biology and drug developments.

1. Introduction

Small molecules have a variety of biological and pharmaceutical functions in living organisms. These compounds, which are natural or artificial, interact with the carrier of genetic information (DNA, RNA) and have wide perspective in chemical genetics, molecular diagnostics and drug development (Stockwell, 2004; Gao et al., 2004). Adenosine as an endogenous small molecule with potent vasodilator and antiarrhythmic activities has received much attention in recent years. In the peripheral nervous system, adenosine is involved in the control of smooth muscle contraction, the regulation of cerebral and ocular blood flow, and is a powerful vasodilator (Phillis, 1989; Portellos et al., 1995; McMillan et al., 1999). In the central nervous system, it plays a well-established modulatory role of neurotransmission and serves as a neuroprotective agent against ischemic- and seizure-induced neuronal injury (Dunwiddie and Masino, 2001). Moreover, adenosine is the core of the cell's energy-containing compound, ATP. The detection of adenosine therefore is of great value. Methods currently reported for the assays of adenosine include affinity chromatography, capillary electrophoresis, electrochemistry, fluorescence, colorimetry, etc. (Deng et al., 2003; Lin et al., 1997; Sun et al., 2009; Wu et al., 2007; Chen et al., 2010; Liu et al., 2009). Though these methods serve as useful tools for the determination of adenosine, the need of precise pretreatment, complicated electrode modification, or limited throughput may pose limitations in their practical applications. Consequently, the development of sensitive, specific, cost-efficient, easily automated for parallel assays of adenosine are greatly desirable.

Signal amplification strategy based on Exo III has been employed to produce stronger optical signal (fluorescence, SPR, UV–vis, etc.) in various DNA sensors (Wang et al., 2005; Ma et al., 2011; Lee et al., 2005; Ou et al., 2010; Chen et al., 2011). Exonuclease III (Exo III) is a DNA-modifying enzyme and is used very frequently in molecular biology. It acts as a 3′–5′ exonuclease liberating 5′-mononucleotides from the 3′-hydroxyl termini of duplex DNA as well as an endonuclease at AP sites. The enzyme's preferred substrates are 3′ blunt or recessed termini of dsDNA and does not attack single-stranded DNA (ssDNA) or oligonucleotides since the hydrolysis is specific for base-paired nucleotides (Richardson et al., 1964; Brutlag and Kornberg, 1972; Zhang et al., 2008; Roychoudhury and Wu, 1977). The cleavage of DNA by Exo III appears to be processive, and cyclic enzymatic digestion of DNA can be achieved. These characteristics provide a useful tool for probing the target selectively in vitro after Exo III treatment (Ren et al., 2004; Zuo et al., 2010).

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Table 1
Synthesized oligonucleotides probes (5′–3′).

Aptamer	ACCTGGGGGAGTATTGCGGAGGAAGGT *TTTT*
Signal probe	FAM-**TATTGCGGAGGAA** C
cDNA 1	**GTTCCTCCGCAAT** TTTT
cDNA 2	**GTTCCTCCGCAATA** CT TTTT
cDNA 3	**GTTCCTCCGCAATA** CTCC TTTT

In the present study, we developed the proof-of-principle of a novel amplified fluorescent aptasensor for adenosine detection employing the cleavage function of exonuclease III of *Escherichia coli* on double-stranded DNA (dsDNA). The sensing mechanism of the approach is based on the following: the adenosine aptamer provides with the capacity of selective binding the target molecules and the Exo III offers the sequence-independent amplification and detection. Compared with existing assay technologies that commonly involve separation steps, surface-based operations or complicated modifications, our assay strategy is able to offer desirable sensitivity, high selectivity, increased throughput, low cost, and simplified operations.

2. Experiments

2.1. Reagents

All the oligonucleotides probes used in the experiments, as shown in Table 1, were obtained from Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). Exonuclease III was purchased from Takara Biotechnology Co. Ltd. (Dalian, China). Graphene oxide (GO) was synthesized by a modified Hummers' method (Hummers and Offeman, 1958; Kovtyukhova et al., 1999). Tris(hydroxymethyl)aminomethane (Tris) and all the other chemicals were of analytical reagent grade and were used as received without further purification. Solutions were prepared with deionized water processed with a Milli-Q ultra-high purity water system (Millipore, Bedford, MA, USA). Unless otherwise stated, all the reagents were prepared with Tris–HCl buffer containing 20 mM Tris, pH 8.0.

2.2. Fluorescence detection of adenosine

The standard solution of adenosine was prepared in Tris–HCl buffer (20 mM Tris–HCl, 100 mM NaCl, pH 8.0). Different concentrations of adenosine were obtained by diluting standard solutions. The following procedure was used to study the concentration-dependent experiments: A 5 μL droplet of aptamer (0.8 μM) was mixed with 10 μL different concentrations of adenosine, the mixture was incubated for 1 h at room temperature. Then, the cDNA2 (5 μL, 0.8 μM) and the signal probe (5 μL, 4 μM) were added respectively in the reaction mixture and incubated for 40 min. Finally, 16 U Exo III was added to perform the digestion reaction in 4 °C refrigerator. After digestion, the reaction was terminated through heating at 75 °C for 5 min. Subsequently, 470 μL GO (6 μg) was added to give final volumes of 500 μL. Then, the fluorescence spectra were measured by a Fluoromax-4 spectrofluorometer (HORIBA Jobin Yvon, Inc., NJ, USA) using ultraviolet quartz cell at room temperature. The emission spectra were recorded in the wavelength range of 503–650 nm upon excitation at 494 nm. Excitation and emission slits were set as 3 nm and 5 nm, respectively.

2.3. Gel electrophoresis analysis

The samples in the absence and presence of adenosine were prepared according to the fluorescence detection procedures. The aptamer, cDNA2, signal probe and cDNA2/signal probe samples without enzymatic digestion were also prepared, and the relevant reagents were replaced by their corresponding buffer solution. 4% (w/v) agarose gels were dissolved in TAE (Tris-HAc 40 mM, and EDTA 1 mM and pH 8.0) buffer. 10 μl of each samples and 2 μl of DNA marker were mixed with 1.5 μl of GelDye TM Super Buffer Mix, and loaded into the gels. The gels were run at 110 V and photographed under UV light using a fluorescence imaging system (Vilber Lourmat, Marne laVallee, France).

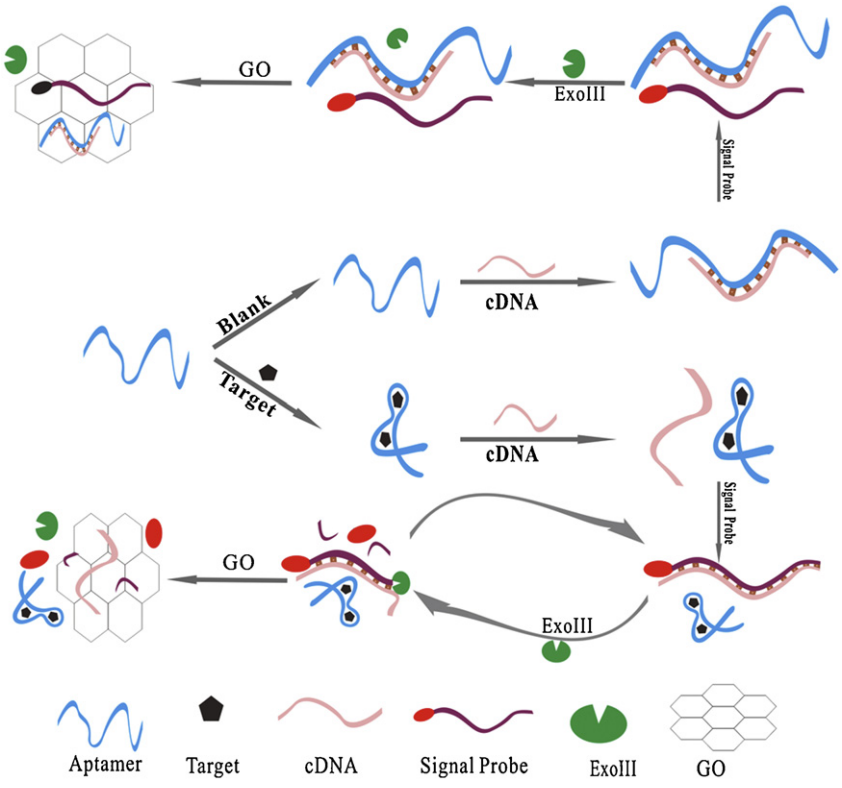
3. Results and discussion

3.1. Design of the fluorescence sensor

Our new detection system consists of Exo III, a single-stranded signal probe, an adenosine aptamer, three complementary DNAs (cDNAs) and the aqueous solution of GO. The binding site of adenosine is the italic region of the aptamer sequence. The bold part of the signal probe is complementary to that of the cDNAs and the underlined sequences of the cDNAs are designed to complement to the partial aptamer sequence. The aptamer and the cDNAs are specially designed and all of them have four thymine nucleotides on their 3′ ends to resist the cleavage of Exo III. Some literatures have reported that double-stranded DNAs with up to three mismatched terminal nucleotides at far recessed 3′ ends were hydrolyzed by Exo III while that of more than 4 nt could not be digested (Brutlag and Kornberg, 1972). The 4 nt-protrusions in the double-stranded DNA with cytosine nucleotide in the 3′ terminal position were a preferred substrate for Exo III (Linxweiler and Horz, 1982; Hoheisel, 1993). Based on this, four thymine nucleotides 3′ overhangs are used to protect DNA terminal from Exo III digestion in order to achieve unidirectional deletion. As illustrated in Scheme 1, the developed aptasensor demonstrated a signal-on architecture in response to the target. In the absence of adenosine, the adenosine aptamer hybridized with the cDNA, and the Exo III could not cleave the single-strand signal probes labeled with carboxylfluorescein (FAM) at its 5′ ends. When the GO was finally added, it could strongly adsorb the single-strand signal probes because of the π-stacking interaction between the ring structures in the nucleobases and the hexagonal cells of the GO and quenched the fluorophore effectively (Lu et al., 2009; He et al., 2010). In the presence of adenosine, the aptamers associated with the targets, which led to the formation of duplex DNAs between the cDNAs and the signal probes. The Exo III thereafter could digest the duplex DNAs from 3′ blunt terminus of signal probes, liberating the fluorophore. The released cDNAs then hybridized with another signal probes to undergo a new cleavage reaction. Through such a cyclic hybridization–hydrolysis process, an adenosine molecule could trigger cleavage of a large quantity of signal probes. Upon adding the GO, the fluorophore could not be adsorbed and quenched, resulting in a remarkable fluorescent increase. Fig. 1 shows the feasibility of the proposed method. An over 100% relative fluorescence signal ($R\,(\%) = (F - F_0)/F_0 \times 100$, where F and F_0 are the fluorescence intensity at 517 nm in the presence and absence of adenosine, respectively. Unless otherwise indicated, the relative fluorescence was used throughout the experiments) increase was observed when the Exo III was employed. However, the corresponding signal gain in the absence of exonuclease III amplification was only 40% or so, which is due to the formation of duplex DNAs between the cDNAs and the signal probes in the presence of adenosine, the GO does not adsorb double stranded DNAs and so the fluorescence of the probe is not quenched.

3.2. Electrophoresis characterization

Direct evidence to show the signal amplification strategy is provided by agarose gel electrophoresis analysis. As shown in



Scheme 1. Schematic representation of the enzymatic amplified assay of adenosine.

Fig. 2, the band of aptamer in lane 1 was too weak to be observed when the gels were run for 30 min. However, there was a vague band when doing it for 10 min, demonstrating the existence of aptamer (Fig. S1). Lane 4 showed lower mobility and brighter than Lane 2 and 3 due to the formation of dsDNA structure. However, there was no band appeared between 20 bp and 40 bp in lane 5 and 6, indicating no dsDNA existed in the samples. Two brighter fluorescent bands were also observed in lane 5 and 6, and the result could be attributed to the enzymatic reaction. It is well known that the fluorophore is very sensitive to the external environment and surrounding nucleotide sequences, and the four nucleotides can more or less quench the fluorophore. Thus, once the single-stranded oligonucleotide labled with fluorophore formed duplex DNA with its cDNA or the fluorophore was liberated from the single-stranded oligonucleotide, the fluorescence would be enhanced to some extent compared with the case mentioned above. In the presence of adenosine, the Exo III could digest the signal probes, liberating the fluorophores. However, the signal probes could also be digested to a certain degree in the absence of adenosine. Therefore, we could observe the brighter bands in Lane 5 and 6 than that of Lane 3. These results demonstrated that the Exo III could successively digest the signal probes, liberating the fluorophores and realizing the amplification of signal successfully.

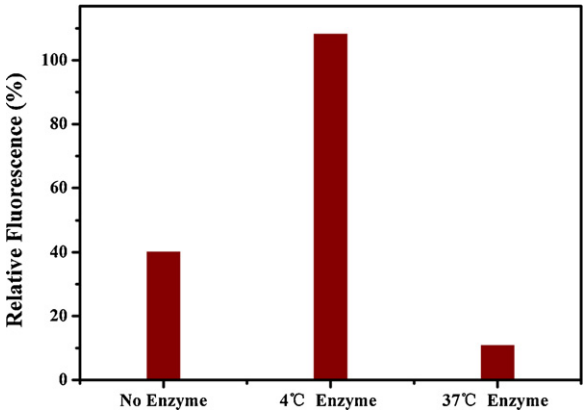


Fig. 1. The relative fluorescence response of the sensing system under the circumstances of no enzymatic cleavage, 4 °C and 37 °C enzymatic cleavage, respectively. The concentration of adenosine is 100 μM. The emission spectra were recorded in the wavelength range of 503–650 nm upon excitation at 494 nm. Excitation and emission slits were set as 3 nm and 5 nm, respectively.

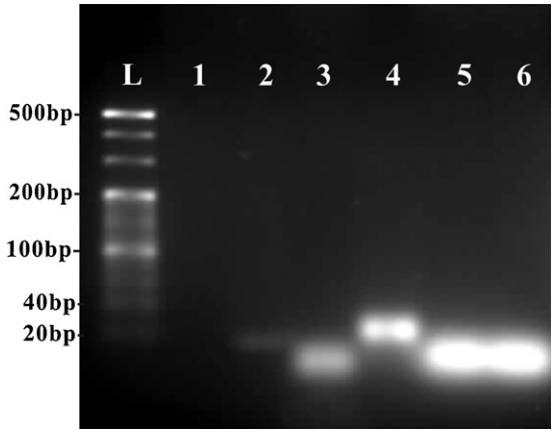


Fig. 2. Gel electrophoresis image for signal amplification strategy. Lanes L, DNA marker; lane 1, aptamer; lane 2, cDNA2; lane 3, signal probe; lane 4, cDNA2/signal probe; lane 5, aptamer/cDNA2/signal probe digested by Exo III; lane 6, aptamer/adenosine/cDNA2/signal probe digested by Exo III; The concentrations and volume of aptamer, cDNA2, signal probe and Exo III are 0.8 μM/5 μl, 0.8 μM/5 μl, 4 μM/5 μl and 8 U/μl/2 μl, respectively. The gels were run for 30 min.

3.3. Optimizing experiment parameters

Reaction temperature is of great importance for the enzymatic cleavage. Theoretically, the optimum incubation temperature should be carried out at 37 °C. However, the net signal gain was very low under these conditions (Fig. 1). We reasoned that the hybridized signal probes on cDNAs were digested by the Exo III. Followed by the enzymatic reaction proceeding, the hybrids of the signal probes and the cDNAs become unstable, inducing the release of fluorophore which has about 7 nt tail sequence accompanied with the dissociation of the hybrids (Okano and Kambarai, 1995). The shortened signal probes were strongly adsorbed by the GO and the fluorescence was quenched, which resulted in a low fluorescence signal in the presence of adenosine. when the assay was conducted at 4 °C, the exonuclease could digest the signal probes into extremely short oligonucleotides. The released quasi-free or free FAM fluorophores could not be adsorbed by the GO, which led to dramatically enhanced signal gain and significantly improved detection limit (Fig. 1).

The incubation time for DNA cleavage by Exo III is another important factor in our experiments. Fig. S2 depicts the cleavage time-dependent response signal for the adenosine assay. The fluorescence signal increased rapidly with the increment of incubation time, then reached a maximum at about 48 h, and finally decreased slowly. We speculated that further increase of the incubation time gave rise to the elevation of fluorescence background because of the single-stranded signal probes were digested by the Exo III (Okano and Kambarai, 1995), hindering the sensitive monitoring of adenosine. Therefore, 48 h was set as the optimal cleavage time. Though the cleavage time estimated by the present assay scheme is longer than previous reports (Wang et al., 2005; Ren et al., 2004; Zuo et al., 2010) due to the low digestion reaction speed at 4 °C, the gain of our assay was prominent that we could readily obtain a remarkable signal difference between the target and blank. Besides, to achieve the maximum fluorescence signal difference, the sequence length of cDNAs (cDNA1, cDNA2 and cDNA3) was also investigated in the absence and presence of 100 μM adenosine. As shown in Fig. S3, the 17 mer cDNA1 showed a moderate signal difference owing to the rather low ability of associating with signal probe for its short sequence. The 20 mer cDNA2 was the most effective oligonucleotide and obviously had fluorescence enhancement efficiency. Further increase of the sequence length (cDNA3) could not augment the difference due to its hybridization with aptamer, inhibiting the cleavage of signal probes. This observation implied that the appropriate length of cDNAs could effectively enlarge the fluorescence response difference and simultaneously maintain the stability of aptamer/adenosine complex. Then the cDNA2 was chosen as the optimum cDNA due to its good performance in the experiments.

3.4. Analytical performance of the aptameric sensor

As a successful protocol for adenosine detection which can be adapted to potential clinical applications, good analytical performance is one of the most important issues. In our proposed strategy, we employed Exo III for the performance enhancing. Under the optimum conditions, we evaluated the fluorescence spectra of exonuclease amplified assay for adenosine of varying concentrations. Fig. 3 displays the fluorescence response to the adenosine concentrations in the range from 0 nM to 100 μM. The peak intensity showed a linear correlation to the logarithm of adenosine concentration within the range from 5 nM to 100 μM (inset of Fig. 3), and the regression equation was $F = 1.30 \times 10^5 \log[C_{adenosine}] + 1.87 \times 10^6$ with a correlation coefficient of 0.99. The detection limit was experimentally determined to be 1 nM. Because the given detection limit in our experiment is not calculated based on theoretical calculation (3σ) but the minimum concentration which can distinguish from the blank sample under the sensor system, The result is of great applicability and operability. Compared with other reported methods of adenosine assays (Lin et al., 1997; Deng et al., 2003; Wu et al., 2007; Sun et al., 2009; Liu et al., 2009; Chen et al., 2010; Huang et al., 2011; Yan et al., 2011), the proposed strategy displayed improved sensitivity. It should be noted that our result was also comparable to the recent reports (Zhou et al., 2010; Zhu et al., 2011; Wang et al., 2011).

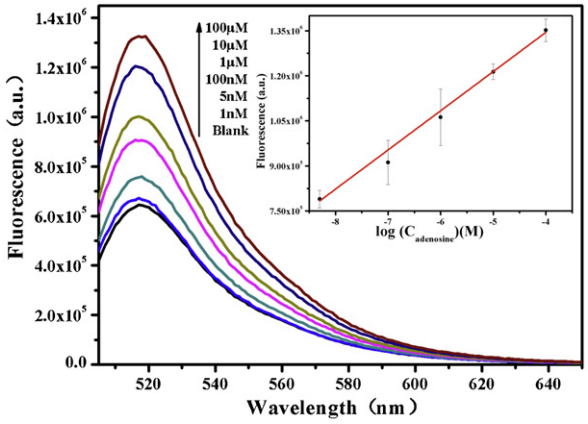


Fig. 3. Fluorescence spectra of exonuclease amplified assay for adenosine of varying concentrations. The inset shows the fluorescence response at 517 nm versus adenosine concentration. Each concentration was measured three times and averaged. Error bars are standard deviation (SD) across three repetitive experiments.

3.5. Selectivity of the aptameric sensor

To verify that the fluorescence signal was triggered by the specific interaction of the aptamers and adenosines, control experiments were also performed by incubating the aptasensor in several aqueous solutions containing uracil, guanine, and cytosine. Fig. 4 depicts the influence of the interferents on the fluorescence change. Although the structure of interferents is similar to that of target, the relative fluorescence response to the interferents was about −5.3–7.0% even at a higher concentration than that of adenosine. Moreover, the fluorescence intensity upon addition of the mixture of adenosine, cytosine, guanine and uracil only decreased slightly, indicating that the fabricated aptasensor is very specific to adenosine.

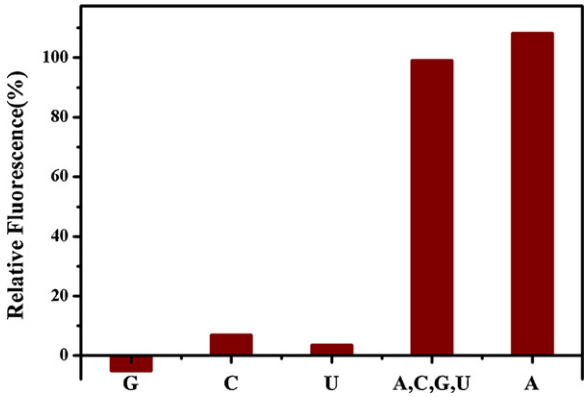


Fig. 4. The relative fluorescence response in the presence of guanine (200 μM), cytosine (200 μM), uracil (200 μM), adenosine (100 μM) and the mixture (uracil (46.8 μM), guanine (46.8 μM), cytosine (46.8 μM) and adenosine (100 μM)), respectively.

4. Conclusion

In summary, we have developed a novel homogeneous fluorescent aptasensor for adenosine assay based on Exo III assisted signal amplification. By coupling cyclic enzymatic cleavage and GO for signal amplification, our system provided a detection limit of 1 nM. More importantly, due to the intrinsic property of Exo III which does not require a specific recognition site and can undifferentiated degrade duplex DNAs from the 3′-hydroxyl termini, our method shows an excellent sequence-independent property and could be widely applied to detect DNAs, proteins, small molecules and metal ions with specific designed oligonucleotides. Notably, the homogeneous assay platform allows this technique to be greatly robust, cost-efficient, readily automated, and scalable for parallel assays of hundreds of samples. In consideration of these advantages, this strategy may hold potential as a versatile platform for molecular diagnostics, chemical biology, and drug developments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2012.01.022.

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