

L-Argininamide biosensor based on S1 nuclease hydrolysis signal amplification

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Abstract A sensitive method is presented for the detection of L-argininamide. It is based on the amplification of the hydrolysis of S1 nuclease of single-stranded regions of an aptamer-target complex. The S1 nuclease, which is sequence-independent, is used to “recycle” target molecules, thus leading to strongly enhanced chemiluminescence (CL). L-Argininamide was chosen as model analyte. The DNA aptamer and its complementary DNA were labeled with the CL reagent N-(4-aminobutyl)-N-ethylisoluminol (ABEI). The DNA complementary to the aptamer was labeled with ABEI and immobilized on magnetic beads (MBs) coated with gold. The aptamer was also labeled with ABEI and self-assembled on the MBs. A duplex was formed due to hybridization between the DNA aptamer and the DNA complementary to the aptamer. In the

presence of the target L-argininamide, a stem-loop aptamer structure is formed which subsequently denatures the duplex. This switch from a duplex structure to a stem-loop structure causes the formation of single-stranded regions both in the target-aptamer and in the single-stranded DNA on the MBs. The nuclease hydrolyzes the single-stranded regions and single-stranded DNA. Ultimately, L-argininamide is released which then interacts with another aptamer on the MB, thereby leading to one more L-argininamide. This autocatalytic cycle can generate substantial quantities of ABEI which then can be sensitively determined by the diperiodatonickelate-isoniazide reaction system. L-argininamide can be detected in the concentration range from 3.0×10^{-4} to 3.0×10^{-7} M, and the limit of detection is 1.0×10^{-7} M.

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Introduction

Aptamers consist of ssDNA, RNA, or modified nucleic acids. Aptamers have recently received considerable attention in chemical-biology research [1, 2]. This kind of DNA molecules, which can be isolated through an in vitro selection process referred to as SELEX (systematic evolution of ligands by exponential enrichment) [3–6], in which aptamers are selected from a library of random sequences of synthetic DNA or RNA by repetitive binding of the oligonucleotides to target molecules [7, 8], are regarded as attractive alternatives to ligands, antibodies and enzymes. Aptamers possess numerous attractive features, including low molecular weight, easy and reproducible synthesis, high binding affinity and molecular specificity [9, 10] fast

tissue penetration and low toxicity [11], tunability in binding affinity, and long-term stability [12]. These advantages have made aptamers an excellent alternative as molecular probes for biomedical studies and clinical applications [13–15]. A large number of DNA aptamers have been produced for the recognition of targets ranging from small molecules to proteins and complex molecular assemblies [16–19].

Detecting small molecules is challenging because the transduction signal in label-free sensors is generally proportional to the mass of the target molecule [20]. To achieve high sensitivity in biosensor, recently, many different methods based on DNA aptamer have been reported such as strand displacement amplification (SDA) [21], aptamer-based machine [22], rolling circle amplification (RCA) [23, 24], nicking endonuclease signal amplification [25].

S1 nuclease is an endonuclease that is active against single-stranded DNA and RNA molecules. Although its primary substrate is single-stranded, it can also occasionally introduce single-stranded breaks in double-stranded DNA or RNA, or DNA-RNA hybrids. Namely, it can hydrolyze single-stranded regions in duplex DNA such as loops and gaps. It is usually used in the laboratory as a reagent in nuclease protection assays. In molecular biology, it is often used in removing single stranded tails from DNA molecules to create blunt ended molecules and opening hairpin loops generated during synthesis of double stranded cDNA [26]. More importantly, S1 nuclease does not require a specific recognition site and 3' to 5' (or 5' to 3') direction of hydrolysis, so hydrolysis occurs irrespective of the sequence and direction present at the terminus or loops. This contrasts sharply with the nicking endonucleases utilized in earlier molecular beacons, nanoparticle amplification schemes or quantum dots coupled fluorescence resonance energy transfer [25, 27–30].

Using the hydrolysis function of S1 nuclease on single-stranded DNA, here we introduce a novelty scheme of signal amplification technology to increase the detection sensitivity of L-argininamide. In this method, detection signal is amplified through aptamer recognition and S1 nuclease hydrolysis, by which one target L-argininamide molecule produce many signal probes. Signal amplification brought about significant increasing in the sensitivity of L-argininamide detection based on S1 nuclease hydrolysis signal amplification and structure-switching aptamer technology. To the best of our knowledge, it is the first time that S1 nuclease hydrolysis signal amplification was used for L-argininamide detection with the merits of being simple and sensitive. Therefore, it is believed that this S1 nuclease hydrolysis signal amplification technique possesses great potential for the highly sensitive detection of biological small molecules, biomarkers and so on.

Experimental

Reagent and instruments

N-(4-aminobutyl)-N-ethylisoluminol (ABEI) was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan. www.tci-asiapacific.com). S1 nuclease ($100 \text{ u}\mu\text{L}^{-1}$) (containing reaction buffer) was obtained from MBI Fermentas (Ontario, Canada. www.fermentas.com). L-argininamide, L-tyrosinamide, L-tyrosine, L-phenylalanine, L-arginine, L-lysine were obtained from Sigma (St. Louis, USA). Fetal bovine serum (FBS) was purchased from Tianjin Haoyang Biological Manufacture Co., Ltd (Tianjin, China. www.tbdsience.com). Isoniazid was obtained from National Institutes for Food and Drug Control (Beijing, China. www.nicpbp.org.cn). Magnetic bead covered with colloid gold (MB) (GoldMag[®]-AS, $5 \mu\text{m}$, $5 \text{ mg}\cdot\text{mL}^{-1}$) were purchased from Shaanxi Lifegen Co.,Ltd. (Xian, China. www.lifegen.com). Magnetic rack was obtained from BaseLine Chrom Tech Research Centre (Tianjin, China. www.qiuhuan.com). All the other reagents were analytical grade and used without further purification.

All synthetic oligonucleotides were purchased from SBS Genetech Co. Ltd. (Beijing, China. <http://www.sbsbio.com/default.asp>). Their sequences were presented as follows:

DNA1, 3'-SH-CTAGCTTCCGCGATGCAAAGCTAG- $\text{PO}_4^{3-5'}$ (complementary to DNA2)

DNA2, 5'-GATCGAAACGTAGCGCCTTCGATC- $\text{PO}_4^{3-3'}$ (L-argininamide aptamer) [41, 42]

CL emission was detected with a FI-CL instrument (IFFM-E, Remex Analytical Instrument Co. Ltd., Xian, China. <http://www.xaremex.com>).

Preparation of ABEI-labeled DNA1/DNA2

ABEI-labeled DNA1/DNA2 (DNA1-ABEI or DNA2-ABEI) were prepared according to the reference with a slight modification [31]. Briefly, $200 \mu\text{L}$ of a $0.1 \text{ mol}\cdot\text{L}^{-1}$ imidazole solution (pH 6.8) was added to the 2 OD (about $66 \mu\text{g}$ of DNA) 5'-phosphate terminal of DNA1 or 3'-phosphate terminal of DNA2 for the activation of the phosphate group for 30 min, then $100 \mu\text{L}$ of $0.1 \text{ mol}\cdot\text{L}^{-1}$ EDAC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) and $200 \mu\text{L}$ of $1 \text{ mmol}\cdot\text{L}^{-1}$ ABEI were added. The labeling reaction was incubated at room temperature for 12 h with shaking. Finally the solution was transferred to a 5 mL centrifuge tube, and $100 \mu\text{L}$ (1/5 volumes) of $3 \text{ mol}\cdot\text{L}^{-1}$ sodium acetate and 2.0 mL (4 volumes) of 100% cold ethanol was added. The solution was chilled for 8 h at $-16 \text{ }^\circ\text{C}$ and then centrifuged for 20 min ($18,000 \text{ rpm}\cdot\text{min}^{-1}$). The

precipitate was washed with 200 μL of cold 70% ethanol several times to remove any free ABEI. Finally, the resulting DNA1-ABEI or DNA2-ABEI was dissolved in water and stored at $-16\text{ }^\circ\text{C}$ for the immobilization and hybridization.

Preparation of MB-DNA1-ABEI conjugates

The MB-DNA1-ABEI conjugates were synthesized by adding 300 μL of the prepared aqueous MB solution (5 mg mL^{-1}) to 100 μL DNA1-ABEI. Thiol-modified DNA1-ABEI was activated with Tri(2-carboxyethyl) phosphine hydrochloride (TCEP, 98%) (10 mM) for 1 h before being attached to MB solution. After shaking gently for 16 h at room temperature, the MB-DNA1-ABEI conjugates were “aged” in the solution (0.3 mol. L^{-1} NaCl, 10 mmol. L^{-1} Tris-acetate, pH 8.2) for another 48 h. Excess reagents were removed by magnetic separation. Following removal of the supernatant, the resulting MB-DNA1-ABEI conjugates were washed with 500 μL of 0.1 mol. L^{-1} pH 7.0 phosphate buffer containing 0.1 mol. L^{-1} NaCl, recentrifuged, and then redispersed in 500 μL of 0.1 M pH 7.0 phosphate buffer containing 0.3 mol. L^{-1} NaCl.

Fabrication of S1 nuclease signal amplification biosensor

The procedure of the fabrication of S1 nuclease signal amplification biosensor and the principle of S1 nuclease signal amplification-based CL detection of L-argininamide are illustrated in Scheme 1. Briefly, 1.0 $\text{mg}\cdot\text{mL}^{-1}$ stock solutions of MB-DNA1-ABEI conjugates, and DNA2-ABEI, were prepared at a concentration of 1.0 mmol. L^{-1} in phosphate buffer solution (pH 7.4). First, 100 μL DNA2-ABEI was incubated with the prepared MB-DNA1-ABEI conjugates and allowed to react in 0.1 mol. L^{-1} phosphate buffer solution for 60 min at $37\text{ }^\circ\text{C}$. After washing with 200 μL of 0.01 mol. L^{-1} phosphate buffer solution three

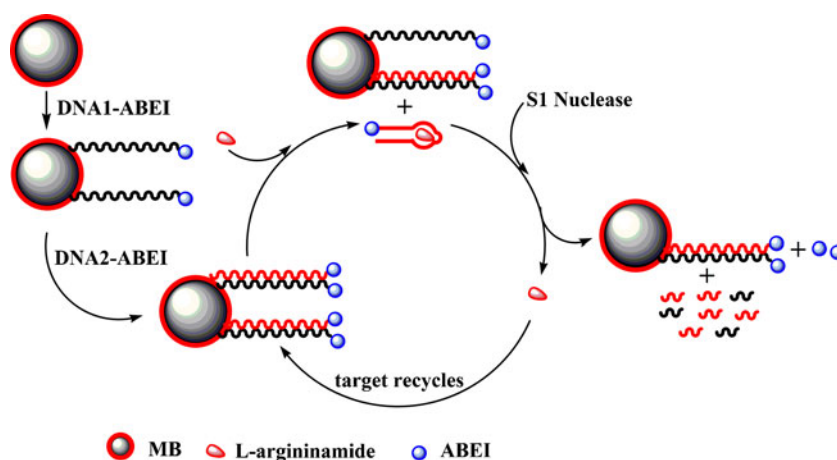
times, various samples (200 μL) at a specific concentration were added and kept for 30 min to make the aptamer change its structure to bind L-argininamide. Then S1 nuclease was added to the solutions and kept for a period of time at $37\text{ }^\circ\text{C}$. After magnetic separation for about 15 min., the supernatant was taken for CL detection. The peak height of intensities was used for quantification. The flow injection system is shown in Scheme S1 (Electronic Supplementary Material; ESM).

Results and discussion

Fabrication of biosensor and detection process

The L-argininamide binding aptamer (L-argininamide-aptamer complex) has a unique structure with stem-loop conformation that allows S1 nuclease hydrolyze single-stranded regions in the DNA. Using the hydrolysis function of S1 nuclease on single-stranded DNA, an amplified L-argininamide detection scheme has been designed. We employed an *in vitro* selected 24-base L-argininamide aptamer, which possesses high affinity for L-argininamide. The DNA1 (L-argininamide aptamer complementary sequence) labeled with 5'-ABEI (DNA1-ABEI) is immobilized on colloid gold covered magnetic beads (MBs). DNA2 (L-argininamide aptamer) labeled with 3'-ABEI (DNA2-ABEI) is self-assembled on MBs in duplex form (Scheme 1). In the presence of the target L-argininamide the stem-loop aptamer structure is formed, which responsively denatures the duplex and liberates from complementary DNA2 [32]. As a consequence of this structural switch from the duplex to the stem-loop aptamer structure, the single-stranded regions in the DNA2 are formed. S1 nuclease catalyzes the stepwise removal of mononucleotides of the single-stranded regions and ultimately releases the L-argininamide. The released L-

Scheme 1 Schematic diagram for the L-argininamide biosensor fabrication based on S1 nuclease hydrolysis signal amplification



argininamide then interacts with another aptamer, whence the cycle starts anew. At the same time, the formed single-stranded DNA on the surface of MB was also hydrolyzed by S1 nuclease. Thus, a single L-argininamide generates many CL reagents ABEI. The large amount of released ABEI could be sensitively determined by the ABEI-DPN-isoniazid [33] (diperiodatonickelate, DPN) reaction system and generated a strong CL signal.

The effect of dose of S1 nuclease on CL intensity

In order to get the optimal analytical parameters, the relative experiments conditions such as incubation time of S1 nuclease and dose of S1 nuclease hydrolysis were first investigated.

S1 nuclease is one kind of endonuclease that digests single-stranded DNA substrate. In the high-concentration, S1 nuclease would also digest the double-stranded DNA (http://en.wikipedia.org/wiki/S1_nuclease). In order to determinate the optimal dose of S1 nuclease in this system, the lowest concentration of S1 nuclease which can digest the double-stranded DNA (a control experimental, hydrolysis of the double-strand with S1 nuclease) (the details information can be seen in Supporting Information) was first investigate. DNA2-ABEI and the prepared MB-DNA1-ABEI were incubated together and allowed to react in 0.1 mol.L^{-1} phosphate buffer solution for 30 min at 37°C . Then S1 nuclease was added to the solutions and kept for a period of time at 37°C . After magnetic separation, the supernatant was taken for CL detection. After magnetic separation, the DNA on the MB would be double-stranded DNA because superfluous DNA2-ABEI was used and no L-argininamide was added. The lower concentration of S1 nuclease should give no CL signal. The experimental results suggested that no obvious signal was observed when the dose of S1 nuclease is lower than $12 \mu\text{L}$. But the CL intensity obviously increased when the dose of S1 nuclease increased over $14 \mu\text{L}$ (Fig. 1(a)). This suggested that the double-strand DNA on the surface of MB can be hydrolyzed by S1 nuclease when the dose of S1 nuclease is higher than $14 \mu\text{L}$. So in the further experimental the dose of S1 nuclease $<12 \mu\text{L}$ was used. In the S1 nuclease hydrolysis signal amplification detection experimental, it was found that the CL intensity increased with increasing dose of S1 nuclease from 1 to $8 \mu\text{L}$. The released ABEI increased with the increasing dose of S1 nuclease, so the CL intensity increased. After that the CL intensity became more flattened. The CL intensity increased greatly when $14 \mu\text{L}$ S1 nuclease was used. It was due to the hydrolysis of the double-strand on MB. It is consistent with the experimental result of hydrolysis of the double-strand with S1 nuclease. As shown in Fig. 1(b), the dose of S1 nuclease was chosen $8 \mu\text{L}$ as the optimal concentration.

The effect of incubation time of S1 nuclease on CL intensity

The effect of incubation time of S1 nuclease on CL intensity was investigated, which was monitored by detecting the change of CL signal with the increasing of incubation time. DNA2 is hybridized with DNA1 on MB surface. After adding S1 nuclease, the single-stranded regions in stem-loop and the formed single-stranded DNA on the surface of MB were hydrolyzed. The ABEI was released. Figure 2 shows CL signal of supernatant. The CL intensity was increased with the increasing time, indicating that S1 nuclease hydrolysis occurred. The hydrolysis reaction time ranged from 5 min to 50 min was investigated. The peak height increased from 5 to 30 min. After 30 min CL intensity increased very slowly. As the aim was to optimize an assay to be both as quick and

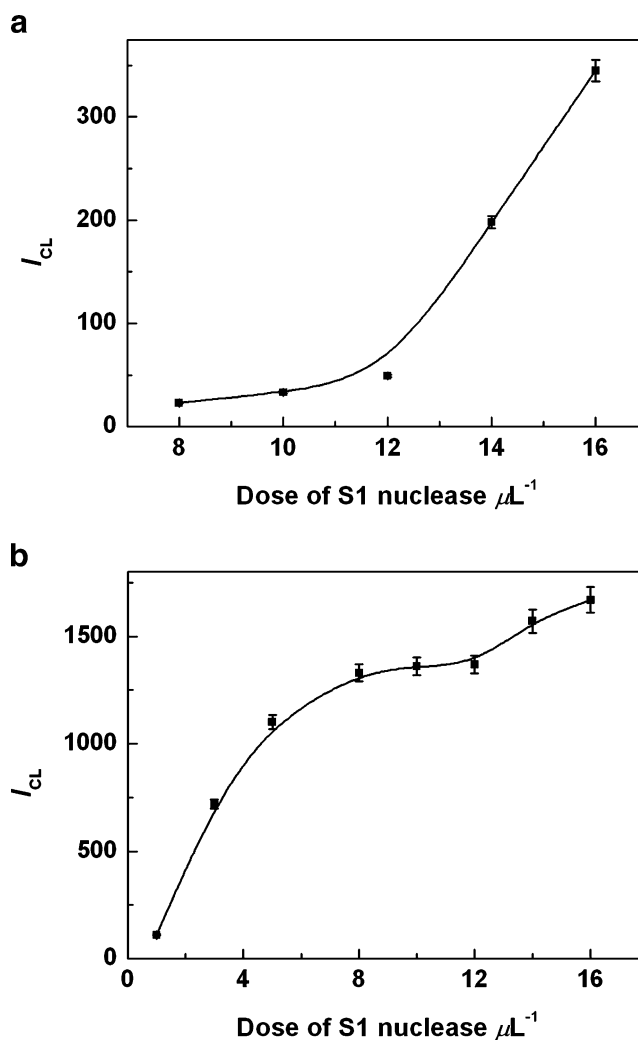


Fig. 1 The effect of dose of S1 nuclease on CL intensity in control experimental (a) and the effect of dose of S1 nuclease on CL intensity in S1 nuclease hydrolysis signal amplification technology (b). The concentration of L-argininamide was $1.0 \times 10^{-5} \text{ mol.L}^{-1}$ in (b)

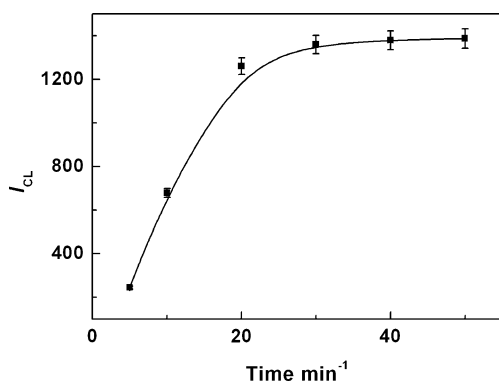


Fig. 2 The effect of incubation time of S1 nuclease with biosensor on CL intensity. The concentration of L-argininamide was 1.0×10^{-5} M. The dose of S1 nuclease was 8 μ L

as efficient as possible, it was decided to perform 30 min incubation in further experiments.

Sensitivity of the L-argininamide biosensor

In the CL detection system, luminol- H_2O_2 -HRP, luminol- H_2O_2 - Co^{2+} [25] luminol- H_2O_2 - Fe^{3+} [34], and 3-(2'-spiroadmantane)-4-methoxy-4-(3''-phosphoryloxy) phenyl-1,2-dioxetane (AMPPD)-alkaline phosphatase [35] are the frequently employed CL systems. In recently years, new CL analytical detection system such as luminol- H_2O_2 -gold colloids [36], CH_3CN -TPA- H_2O_2 - FeCl_3 [37], luminol- H_2O_2 -HB and so on [38, 39] have been developed (The luminol can change as ABEI in these CL systems). The sensitivity of these CL systems is restricted because of the high detection limits of luminol or ABEI. In order to increase the sensitivity of target, a new CL detection system, ABEI-DPN-isoniazid (diperiodatonickelate, DPN) reported by Yang, was used [32].

Based on the combination of the remarkable S1 nuclease hydrolysis signal amplification technology with sensitivity of the ABEI-DPN-isoniazid CL detection system, under the optimized experimental conditions, the CL intensities increased with the increase of the concentrations of L-argininamide ranging from 3.0×10^{-4} to 3.0×10^{-7} M. The nonlinear function for L-argininamide was $I_{\text{CL}} = -81.33 +$

$159.85C - 0.79C^2$ (I_{CL} is the CL intensity; C is the concentration of L-argininamide, μM ; $N=12$, $R^2=0.9995$). The linear range for L-argininamide was 3.0×10^{-7} to 3.0×10^{-5} M with the equation of $I_{\text{CL}} = 135.3C - 32.2$ (I_{CL} is the CL intensity; C is the concentration of L-argininamide, μM ; $N=6$, $R^2=0.9998$) and the detection limit of 1.0×10^{-7} mol.L $^{-1}$ L-argininamide estimated using 3σ . A series of seven repetitive measurements of 1.0×10^{-5} mol.L $^{-1}$ L-argininamide were used for estimating the precision, and the relative standard deviation (RSD) was 3.9%. It was shown that this biosensor had good reproducibility. This method and some other amplified techniques, which can greatly improve the sensitivity of the L-argininamide assay, are listed in Table 1.

A control experiment without employing S1 nuclease hydrolysis signal amplification technology (non-S1 nuclease hydrolysis signal amplification technology) was carried out to further determine the sensitivity of this L-argininamide assay strategy (schematic diagram for non-S1 nuclease hydrolysis signal amplification technology as control experimental can be seen in Scheme S3, ESM). The linear range for L-argininamide was achieved from 1.0×10^{-3} to 6.0×10^{-5} M. The experimental results indicated that S1 nuclease hydrolysis signal amplification technology give approximately a 200-fold improvement in detection sensitivity compared to non-S1 nuclease hydrolysis signal amplification technology (we choose the lowest concentration of the linear range as the comparison standard).

Another control experiment employed ABEI- H_2O_2 - Co^{2+} for the CL detection in S1 nuclease hydrolysis signal amplification technology was also carried out to further determine the sensitivity of this L-argininamide assay strategy (named as S1 nuclease hydrolysis signal amplification coupled with ABEI- H_2O_2 - Co^{2+} CL detection system. The details information about the experiments can be seen in Supporting Information). Under the optimal condition, the linear range for L-argininamide was achieved from 1.0×10^{-3} to 5.0×10^{-6} mol.L $^{-1}$ when ABEI- H_2O_2 - Co^{2+} system was employed in S1 nuclease hydrolysis signal amplification technology. It suggested that the ABEI-DPN-isoniazid system give approximately a 17-fold improvement in

Table 1 Comparison between the current method and other reported techniques for analysis of L-argininamide

Assay format	Detection method ^a	Detection range	Detection limit ^c	Ref
Ionic liquid used as the CE selector	EC	^b	5×10^{-6}	[41]
Ionic liquid used as the CE selector	EC	^b	2×10^{-5}	[42]
Noncompetitive fluorescence polarization	FP	1.0×10^{-3} – 1.0×10^{-5}	^b	[43]
S1 Nuclease hydrolysis signal amplification technology	CL	3.0×10^{-4} – 3.0×10^{-7}	1.0×10^{-7}	This method

^a CE electrophoresis; EC electrochemical; FP fluorescence polarization; CL chemiluminescence

^b Do not reported in their paper

^c mol L $^{-1}$

detection sensitivity compared to ABEI-H₂O₂-Co²⁺ system. All these experimental results confirmed that the ABEI-DPN-isoniazid system have the higher sensitivity. And S1 nuclease hydrolysis signal amplification technology coupled with ABEI-DPN-isoniazid CL detection system can give the sensitivity of detection L-argininamide. Another reason we selected ABEI-DPN-isoniazid as the CL detection system was that this detection system has strong anti-interference ability. 1000 for K⁺, Na⁺, Ca²⁺, Cl⁻, SO₄²⁻, NO₃⁻, starch, carbamide; 500 for Fe³⁺, Cu²⁺, Cr³⁺, Co²⁺, Zn²⁺, lactic acid; 200 for lactose, glucose, uric acid; 100 for citrate; 50 for ascorbic acid have no interference on this CL system. But these substances, such as Fe³⁺, Cu²⁺, Cr³⁺, Zn²⁺, and uric acid, containing in biological sample usually interfere the ABEI-H₂O₂-Co²⁺ system. These intrinsic advantages make this method the practical applicability of real sample.

The reason for this signal amplification can be explained as follows. In the S1 nuclease hydrolysis signal amplification technology, the target L-argininamide binds to its aptamer forming the L-argininamide-aptamer complex and moves away from DNA1. The S1 nuclease hydrolyzes the single-stranded regions in L-argininamide-aptamer complex and the formed single-stranded DNA on the surface of MB. The target L-argininamide is released and acts as recognized element again and circular template for fresh CL probe ABEI giving rise to amplification of detection signal. And one target L-argininamide molecule could generate many CL ABEI probes. According to the amplification ratio of the detection limit, recycling use of L-argininamide is about 200 times in this system.

Specificity of the L-argininamide biosensor

The biosensor specificity was subsequently investigated. To assess the specificity of the method for the detection of L-argininamide, experiments were conducted on enantiomer D-argininamide, aptamer relative substance such as L-tyrosinamide, and closely related compounds L-tyrosine and L-phenylalanine, L-leucinamide. Different CL signals of this system for the detection of 1.0×10^{-5} mol.L⁻¹ D-argininamide, L-tyrosinamide, L-tyrosine, L-phenylalanine and L-leucinamide, were recorded under the same experimental conditions according to the protocol described in experimental section, respectively. On the basis of S1 nuclease signal amplification technology for the detection of D-argininamide, L-tyrosinamide, L-tyrosine, L-phenylalanine or L-leucinamide did not induce any significant changes in the CL signal as compared to that of L-argininamide. As reported in Fig. 3, this biosensor was able to discriminate against D-argininamide, L-tyrosinamide, L-tyrosine, L-phenylalanine and L-leucinamide. It suggests that the developed strategy has a sufficient selectivity and L-argininamide could be unequivocally identified. This may be

explained by the fact that the aptamer change its structure to bind L-argininamide.

The developed S1 nuclease hydrolysis signal amplification technology has the advantage of being significantly simpler than other reported nuclease-based methods. For example, there is no need for multiple enzymes and labeled-tag, as are required in nicking enzyme-based and molecular beacons-based amplification assays [29, 40]. Because the preparation of biosensor could be done before the detection experiment the total detection time of this method is about 1.5 h, which was competitive compared to the methods with a similar signal amplification technology [24, 33]. Only needing a signal probe and two kinds of DNA, the direct detection of target L-argininamide could be accomplished with one step, so the low cost and simple operation was another merit compared to other methods. More importantly, S1 nuclease does not require a specific recognition site and 3' to 5' (or 5' to 3') direction of hydrolysis, it suggests that the present S1 nuclease hydrolysis signal amplification methodology can be potentially generalized. The real samples application of this method and the nanoparticles used as the signal probe were under going.

Conclusions

In summary, we have demonstrated that the present signal amplification method can be applied to the sensitive and selective detection of L-argininamide. By the S1 nuclease hydrolysis signal amplification technology, the detection limit of 1.0×10^{-7} M L-argininamide was obtained. This amount corresponding to about 200-fold of sensitivity achieved compared to non-S1 nuclease hydrolysis signal amplification technology. The L-argininamide assay also exhibits high specificity. Only needing a signal probe and two kinds of DNA, the direct detection of target L-

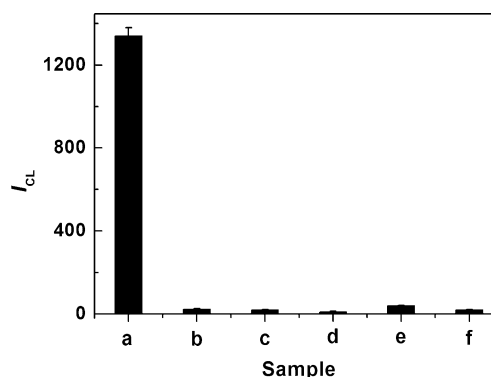


Fig. 3 CL responses for different samples. **a** L-argininamide; **b** D-argininamide; **c** L-tyrosinamide; **d** L-tyrosine; **e** L-phenylalanine; **f** L-leucinamide. The dose of S1 nuclease was 8 μL. The concentrations of samples were 1.0×10^{-5} M. The blank was deducted

argininamide could be accomplished with one step less than 1.5 h. Moreover, S1 nuclease does not require a specific recognition site and 3' to 5' (or 5' to 3') direction of hydrolysis. This simple, low-cost, and highly sensitive method should contribute significantly to future and can be potentially generalized.

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