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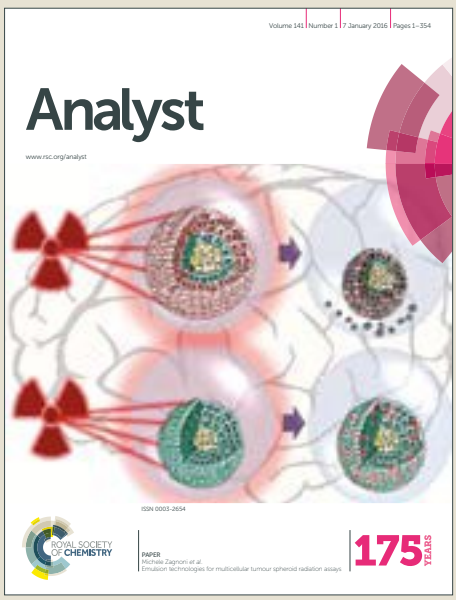

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DOI: 10.1039/C8AN02308E

PAPER

Silver-Ion-Mediated Mg^{2+}-dependent DNAzyme activity for amplified fluorescence detection of cysteine

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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In this paper, by taking advantage of the fact that silver ion could mediate the Mg^{2+}-dependent DNAzyme (Mgzyme) activity, we for the first time developed a turn-on fluorescent biosensor for cysteine (Cys) amplified detection. Because Mgzyme can interact with silver ion and form cytosine-Ag^{+}-cytosine (C-Ag^{+}-C) base pairs, the conformation of its catalytic core was changed. As a result, the catalytic activity of Mgzyme was suppressed and the Mgzyme-Ag^{+} complex could not initiate the cleavage reaction. Therefore, the background fluorescence of biosensor was very low. In the presence of Cys, Cys can bind tightly silver ion and disrupt the C-Ag^{+}-C base pairs in the Mgzyme-Ag^{+} complex, leading to the restoration of Mgzyme activity. The activated Mgzyme could hybridize with MB substrate and undergo many cleavage cycles, resulting in a significant increase of fluorescence intensity. This designed strategy provided an amplified fluorescence detection of cysteine, with a detection limit of 2 nM. Moreover, the strong binding between Cys and Ag^{+} ensured that the biosensor had a desirable selectivity for Cys. This sensing system was also used to detect Cys in human urine samples and displayed satisfying results.

Introduction

DNAzymes are a kind of nucleic acids which can be obtained successfully through in vitro selections and have remarkable catalytic cleavage activity toward ribonucleobase(rA)-containing substrate sequences by utilizing some specific metal ions or molecules as cofactors.[1-4] What's more, DNAzymes possess several unique properties in comparison with protein enzymes, such as easier synthesis and modification, lower production price and higher stability to resist hydrolysis and denaturation.[5-7] Due to these excellent properties, DNAzymes have attracted a great deal of research interest in recent years. Some DNAzyme-based biosensors have been designed to detect kinds of cofactors, including metal ions,[8-10] small molecules[11,12] and proteins[13] and so on.[14] Besides, DNAzymes were also used as effective amplifying labels to construct nonprotein-enzymatic amplified sensing platforms for DNA, proteins and other targets detection.[15-24] For example, Zhang group previously designed a versatile amplified assay platform for different targets detection by taking advantage of the topological effect of Zn^{2+}-dependent DNAzyme (Znzyme).[24] Wei et al. developed a colorimetric sensor for the detection of protein utilizing the assembly of Mgzyme.[15] However, most of DNAzyme-based amplified sensing systems were based on the design strategy that the target recognition units must be covalently connected with the DNAzyme sequences, which need complicated design and high cost. Moreover, some targets such as Cys are not fit for this working mechanism, because it is difficult for some target recognition units to integrate with DNAzyme sequences. Therefore, a novel design strategy is required for DNAzyme-based amplified assay platform.

Cysteine (Cys) is a thiol-containing amino acid and plays a significant biological role in living system by involving kinds of crucial cellular functions, including protein synthesis, metabolism and detoxification.[25-28] The abnormal levels of Cys in biological systems will lead to some diseases.[29-32] For example, the deficiency of Cys is linked to hair depigmentation, liver damage and slow growth and so on,[29] while excess accumulation of Cys is associated with neurotoxicity.[31] Therefore, the detection of Cys concentration in biological fluids is very significant. In the past few years, several methods have been designed for Cys detection, including capillary electrophoresis mass spectrometry[33,34] and high-performance liquid chromatography (HPLC) etc.[35-38] These traditional methods all provide reliable detection results for Cys. However, most of them are usually expensive, time consuming and require sophisticated instrumentation. Very recently, fluorescent detection of Cys has drawn considerable attention owing to its low cost, high sensitivity and easy measure.[39-49] For instance, some biosensors were constructed for Cys detection based on Ag^{+}-mediated conformation change of G-quadruplex.[40,41] In addition, some fluorescent

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[c] Electronic supplementary information (ESI) available. See DOI: 10.1039/x0xx00000x

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nanomaterials such as silver nanoclusters and carbon dots were also synthesized and used for Cys determination.[45] Though these methods are simple and have desirable sensitivity and selectivity for Cys, an even higher sensitivity is needed if we look forward to using the sensor in the early diagnosis of Cys-related diseases.

To develop a highly sensitive biosensor for Cys determination, we turn our attention to metal-dependent DNAzymes on account of their catalytic amplification performance. Here we found that silver ion could suppress the catalytic activity of Mgzyme, which was due to the conformation change of its catalytic core caused by the formation of C-Ag^+-C base pairs. However, the addition of Cys could make the Mgzyme activity recover because Cys can bind tightly silver ion and disrupt the C-Ag^+-C base pairs.[49] Therefore, by taking advantage of the fact that silver ion can mediate the activity of Mgzyme, we for the first time developed a fluorescent turn-on biosensor for Cys detection. A catalytic and molecular beacon (CAMB) strategy that can realize the true multiple enzymatic turnovers was further used to enhance the sensitivity of our proposed biosensor.[50] Moreover, the strong binding between Cys and Ag^+ ensured that the biosensor had a desirable selectivity for Cys. It was also used to detect Cys in human urine samples and displayed satisfying results.

Experimental

Reagents and apparatus

The MB (5'-FAM-CCACAACATTCAAATTCAGGAACTATrAGGAAGAGATGTTACGAGGGGTTGTGG-BHQ1-3'), the Mgzyme (5'-CATCTCTTCAGCGATACTACTACTACTACTTTTTAGTAGTAGTAGTAGTAGTCACCCATGTATAGTTCCT-3') and the Znzyme (5'-CATCTCTTCTCCGAGCCGGTCGAAATAGTTCCT-3') were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Cysteine (Cys), Tryptophan (Trp), Alanine (Ala), Leucine (Leu), Glycine (Gly), Methionine (Met), Tyrosine (Tyr), Isoleucine (Ile), Aspartic acid (Asp), Lysine (Lys), Proline (Pro), Serine (Ser), Glutamine (Gln), Histidine (His), Threonine (Thr), Phenylalanine (Phe), Valine (Val), Asparagine (Asn), Arginine (Arg), Glutamic acid (Glu) were purchased from J&K Scientific Ltd. Metal salts ($AgNO_3$, $Al(NO_3)_3$, $Pb(NO_3)_2$, $Cu(NO_3)_2$, $NaNO_3$, $Mg(NO_3)_2$, $Zn(NO_3)_2$, KCl, $CaCl_2$) and HEPES were obtained from Solarbio Co., Ltd (Beijing, China) and used without further purification. Ultrapure water (Millipore, 18 M Ω /cm) was used throughout the experiment.

The fluorescence measurements were performed on a Hitachi F-4500 Fluorescence Spectrometer (Tokyo, Japan) and the emission spectra were measured from 510 nm to 650 nm. The pH measurement was performed on a Mettler-Toledo Delta FE28 pH meter.

Procedure for the effect of metal ions on the DNAzyme activity

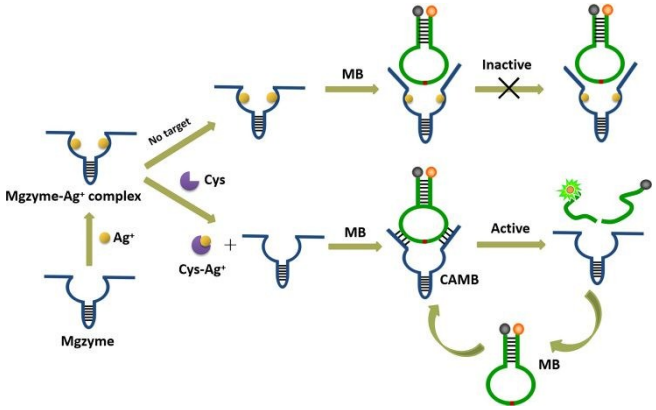
First, 100 nM DNAzme and different metal ions (1 μM) were mixed and incubated for 10 min in HEPES buffer (25 mM HEPES, 100 mM $NaNO_3$, 10 mM $Mg(NO_3)_2$, pH 7.2). Then, 100 nM MB was added into the above solution and reacted for 30 min to detect the fluorescence Intensity changes of the CAMB system.

Procedure for Cys detection

The detection experiment of Cys was carried out in 100 μL HEPES buffer (25 mM HEPES, 100 mM $NaNO_3$, 10 mM $Mg(NO_3)_2$, pH 7.2). First, 50 nM 100 nM MB and 600 nM Ag^+ were mixed and incubated for 10 min in HEPES buffer at room temperature. Then, 100 nM MB and a varying concentration of Cys (in the range of 0-8 μM) were added into the above mixture. After reaction for 30 min, the fluorescence intensity at 518 nm with an excitation wavelength at 494 nm was recorded.

All experiments were performed in accordance with the Guidelines of the relevant institutional authorities, and approved by the ethics committee at Shanxi Medical University. Informed consents were obtained from human participants of this study.

Results and discussion



Scheme 1. The designed strategy of Mgzyme-based fluorescent amplified biosensor for Cys detection.

Sensing principle

The designed strategy of Mgzyme-based fluorescent biosensor for amplified determination of Cys is illustrated in Scheme 1. Since Mgzyme can interact with silver ion and form C-Ag^+-C base pairs, the conformation of its catalytic core were changed. The possible conformations of Mgzyme-Ag^+ complex were shown in Figure S1. In such case, the catalytic activity of Mgzyme was suppressed and the Mgzyme-Ag^+ complex could not initiate the cleavage reaction, which provided a very low fluorescence background for this biosensor. In the presence of Cys, Cys could bind tightly silver ion and disrupt the C-Ag^+-C base pairs in the Mgzyme-Ag^+ complex, leading to the restoration of Mgzyme activity. The activated Mgzyme is hybridized with MB to form the CAMB and triggered the catalytic cleavage of MB by utilizing Mg^{2+} as a cofactor. After cleavage, the Mgzyme was released and the quenched fluorophore/ quencher pair of MB substrate was separated, resulting in an obvious enhancement of fluorescent intensity.

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Eventually, the released Mgzyme could continuously hybridize with another MB substrate and undergo many cleavage cycles, realizing an amplified fluorescence detection of Cys.

The effect of metal ions on the catalytic activity of DNAzyme

In order to survey the effect of metal ions on the catalytic cleavage activity of Mgzyme, the fluorescent intensity of the CAMB system were recorded upon adding different cations (Ag^+, K^+, Zn^{2+}, Cu^{2+}, Pb^{2+}, Ca^{2+}, Al^{3+}). As shown in Fig. 1(A), only Ag^+ induced an obvious fluorescence quenching in comparison with other metal ions. Furthermore, the effect of different concentration of Ag^+ on the catalytic activity of Mgzyme were also investigated. As can be seen from Fig.1(B), the fluorescence signal of CAMB system decreased dramatically as the increasing concentrations of Ag^+. When the Ag^+ concentration reached 1 μ M, the fluorescence intensity was quenched to a level similar to the background fluorescence of the MB substrates. While the fluorescence intensity of the CAMB system based on Znzyme reduced a little upon the addition of 1 μ M silver ion (see the Supporting Information, Figure S2). These experimental results indicated that the catalytic activity of Mgzyme could be regulated by Ag^+ with high selectivity.

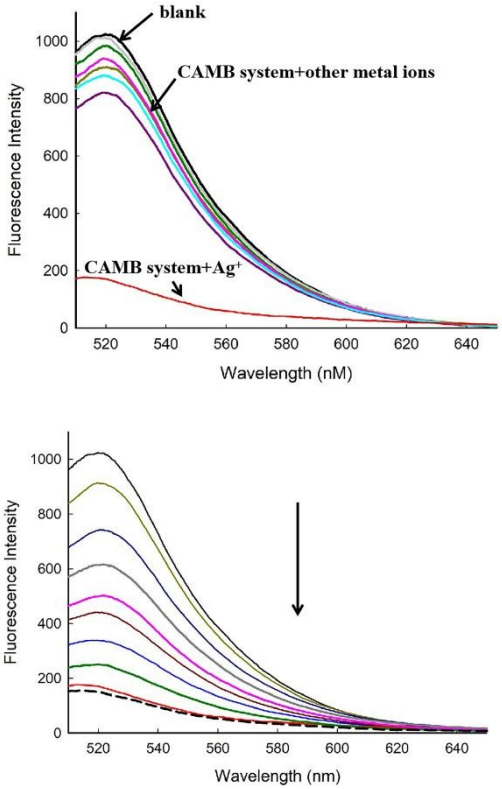

Fig. 1A. The effect of different metal ions (Ag^+, K^+, Zn^{2+}, Cu^{2+}, Pb^{2+}, Ca^{2+}, Al^{3+}) on fluorescent intensity of CAMB system, the concentrations of metal ions were 1 μ M each. **Fig. 1B.** The effect of different concentrations of Ag^+ (0 nM, 100 nM, 200 nM, 300 nM, 400 nM, 600 nM, 700 nM, 800 nM, 1 μ M) on fluorescent intensity of CAMB system in HEPES buffer (25 mM HEPES, 100 mM $NaNO_3$, 10 mM $Mg(NO_3)_2$, pH 7.2). The black imaginary line (---) represented the background fluorescence of the MB. The concentrations of MB and Mgzyme were 100 nM, respectively.

The feasibility analysis of the biosensor

To evaluate the feasibility of the biosensor for Cys detection, the Cys-induced fluorescence intensity changes were recorded in the presence of 10 mM Mg^{2+}. As shown in Fig. 2, the addition of silver ion triggered obvious fluorescence quenching of the sensing system (curve a) and the fluorescence intensity was close to the background fluorescence of the MB substrates (curve b), which demonstrated that the catalytic activity of Mgzyme was suppressed almost completely by Ag^+. Nevertheless, a significant fluorescence enhancement was observed with the addition of target Cys (curve c), which was due to the restoration of Mgzyme activity caused by the strong binding between silver ion and Cys. The results demonstrated the design feasibility of our proposed biosensor.

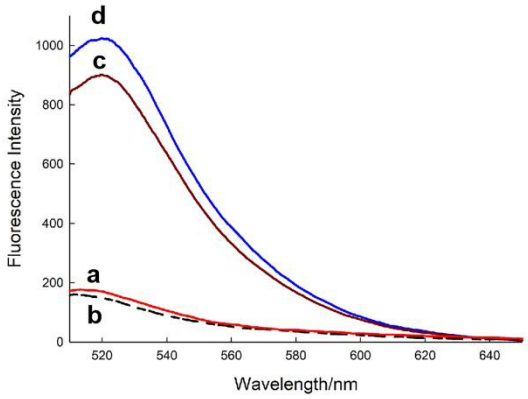

Fig. 2. Fluorescence intensity of the biosensor under different assay conditions in the presence of Mg^{2+} (10 mM): (a) MB+Mgzyme+Ag^+; (b) MB; (c) MB+Mgzyme+Ag^++Cys; (d) MB+Mgzyme. The concentrations of MB substrate and Mgzyme were 100 nM, respectively. The concentration of Ag^+ was 1 μ M and the concentration of Cys was 2.5 μ M.

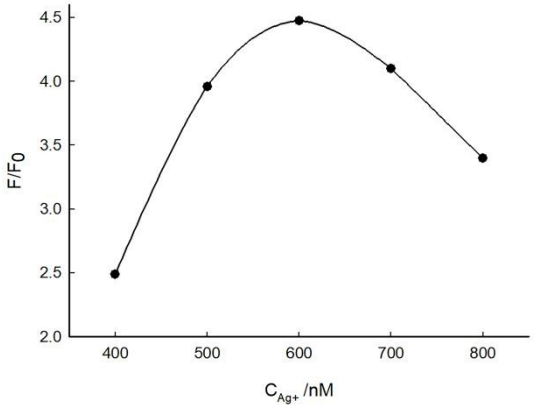

Fig. 3. The effect of Ag^+ concentration on the fluorescence intensity ratio (F/F_0) of the sensing system; the concentrations of Mgzyme and MB substrate were 50 nM, respectively. F and F_0 are the fluorescence intensity of the biosensor in the presence and absence of 2.5 μ M Cys, respectively.

Optimization of assay conditions

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To obtain the best sensing performance, several crucial assay conditions were investigated. Firstly, the concentration of Ag^+ was optimized. As shown in Fig. 3, the fluorescence intensity ratio (F/F_0) of the biosensor increased notably along with the increasing concentrations of silver ion. However, when the concentration of Ag^+ was higher than 600 nM, F/F_0 decreased gradually, which might be caused by excessive suppressive effect of Ag^+ on the catalytic activity of Mgzyme. Therefore, we chose 600 nM silver ion as the optimum concentration in the experiments. Next, the ratio of MB substrate to Mgzyme was further investigated and the assay results were shown in Fig. 4. The F/F_0 of the biosensor enhanced continuously with an increasing ratio of MB substrate to Mgzyme until the ratio achieved 2:1. Nevertheless, when the ratio surpassed 2:1, the F/F_0 of the biosensor reduced with further increasing ratio of MB substrate to Mgzyme on account of the increased background fluorescence of MB substrate. Based on this result, a ratio of MB substrate to Mgzyme at 2:1 was selected as the optimal ratio for further experiments. Last, the concentration of Mg^{2+} was also optimized. Experimental results indicated that 10 mM Mg^{2+} could provide maximum fluorescence intensity ratio for the sensing system (see Supporting Information, Figure S3).

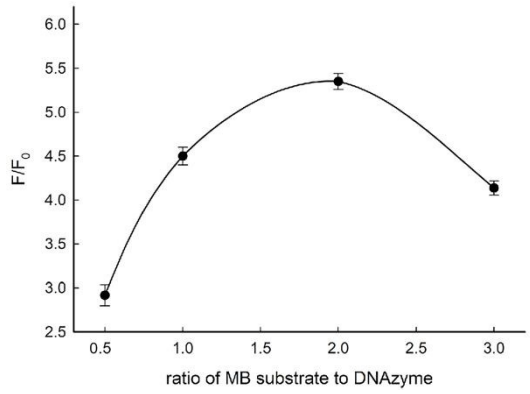

Fig. 4. The effect of the ratio of MB substrate to Mgzyme on the fluorescent intensity of the sensing system, the Mgzyme concentration was fixed at 50 nM. The F and F_0 are the fluorescence intensity of the biosensor in the presence and absence of 2.5 μ M Cys, respectively.

Sensitivity of the sensing system

To verify the sensitivity of the biosensor, the fluorescence signal in the presence of various concentrations of Cys were measured under optimized assay conditions. As can be seen from Fig.5A, in the absence of Cys, a low initial fluorescence signal was observed because of the effective suppressive effect of Ag^+ on the catalytic activity of DNAzyme. However, the fluorescence intensity of the biosensor enhanced obviously with increasing concentrations of Cys, which might be caused by the fact that the strong binding between silver ion and Cys disrupted the C-Ag^+-C base pairs in the Mgzyme-Ag^+ complex and the Mgzyme activity was recovered. When the Cys concentration increased to 8 μ M, the fluorescence intensity nearly reached a plateau. Fig.5B described the calibration curve of the biosensor and a linear relationship ranging from 5 nM to 500 nM for Cys quantitative detection was observed. The detection limit (based on 3 δ /slope) was 2 nM, which was lower than those of previously reported Cys biosensors.[39-42] Comparison of detection performance of some Cys biosensors were shown in Table 1. The excellent sensitivity of the designed sensing system was due to the low background fluorescent signal of the biosensor and the multiple enzymatic turnovers of the activated Mgzyme. These results indicated that our designed method was fit for quantitative detection of Cys.

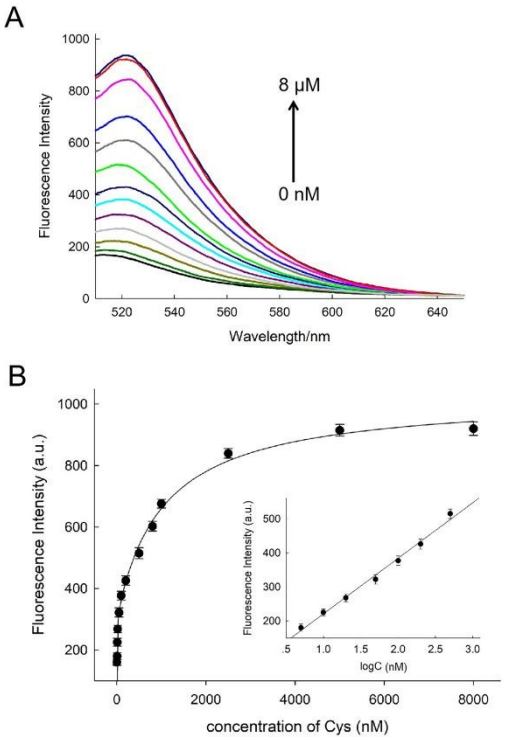

Fig. 5A. The fluorescent intensity of biosensor upon introduction of different concentrations of Cys (from bottom to top: 0, 5 nM, 10 nM, 20 nM, 50 nM,100 nM, 200 nM, 500 nM, 800 nM, 1 μ M, 2.5 μ M, 5 μ M, 8 μ M. **Fig. 5B**. Calibration curve for Cys detection. Inset: the biosensor responses to low concentrations of Cys. The buffer contains 25 mM HEPES, 100 mM NaNO3, 10 mM Mg(NO3)2, pH 7.2.

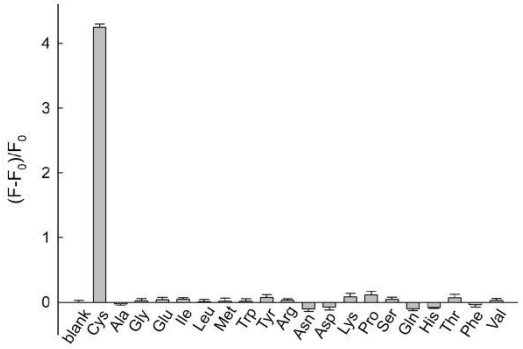

Fig. 6. Selectivity test for Cys. The concentration of Cys was 2.5 μ M , and the concentrations of other amino acids were 25 μ M .

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Table 1. Comparison of detection performance of various Cys biosensors

Technique	Method	LOD	Linear range	Reference
Colorimetry	G4/hemin/turn-off	25 nM	25 nM-2200 nM	[40]
Colorimetry	G4-DNAzyme/turn-off	40 nM	40 nM-110 nM	[41]
luminescence	G4/Tb/turn-off	20 nM	40 nM-1.6 μM	[42]
Fluorescence	L[1]-Cu^{2+}/turn-on	0.19 μM	1 μM-8.0 μM	[44]
Fluorescence	SGI[2]/C-DNA/turn-off	4.5 nM	8 nM-550 nM	[39]
Fluorescence	carbon dots/turn-on	30 nM	0-700 nM	[45]
Fluorescence	Ag^{+}-Mgzyme/turn-on	2.0 nM	5 nM-500 nM	This work

[1]bi-8-carboxamidoquinoline derivative ligand

[2]SYBR Green I

Selectivity of the sensing system

To evaluate the selectivity of our designed sensor, The effects of other 19 α -amino acids were researched by immobilizing the concentration of Cys at 2.5 μ M and other amino acids at 25 μ M. As shown in Fig.6, only Cys induced an obvious fluorescence increasing, while other amino acids had no or very little impact on Cys detection. Although other amino acids could be combined with silver ion as well, their interactions were not strong enough to disrupt the C-Ag^{+}-C base pairs in the Mgzyme-Ag^{+} complex. Therefore, our designed sensor can be used to detect Cys selectively.

Real sample analysis of the sensing system

To evaluate the feasibility and reliability of the sensing system for practical application, the recovery tests with spiked Cys in human urine samples were performed. The urine samples were got from healthy adults and showed that no Cys was present. Firstly, the urine samples were diluted 100 times with the HEPES buffer solution. Then, using a standard addition method, different concentrations of Cys (0.05 μ M, 0.1 μ M, 0.5 μ M) were added to the urine samples and measured directly. As listed in Table 2, the recovery of added Cys was in the range of 94%-103%. These results demonstrated that the developed method could be used to detect Cys in real samples.

Table 2 Recovery experiments of Cys in urine

Sample	Cys spiked (μM)	Cys recovered (μM)	Recovery (%)
urine 1	0.05	0.047[a]±0.004[b]	94
urine 2	0.1	0.103[a]±0.003[b]	103
urine 3	0.5	0.485[a]±0.005[b]	97

[a] Mean of three determinations. [b] SD: standard deviation.

Conclusions

Herein, by taking advantage of the fact that silver ion can mediate the Mgzyme activity, we successfully constructed a turn-on fluorescent biosensor for Cys amplified detection. The low background fluorescent signal of the biosensor and the multiple enzymatic turnovers of the activated Mgzyme made the sensing system exhibit the excellent sensitivity for cysteine, with a detection limit of 2 nM. In addition, the strong binding between Cys and Ag^{+} ensured that the biosensor had a desirable selectivity for Cys. Moreover, the sensing system was used to detect Cys in human urine samples and showed satisfying results.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (21405100, 21705043). The Doctoral Startup Research Fund of Shanxi Medical University (03201306, 03201308), Student's Platform for Innovation and Entrepreneurship Training Program (2018192).

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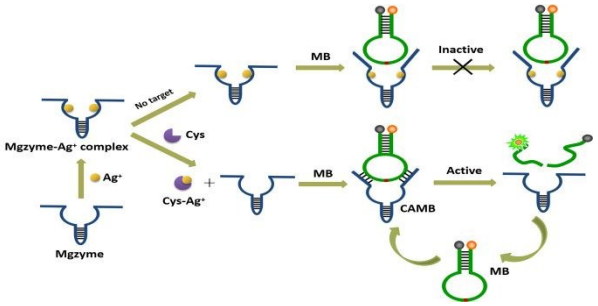
DOI: 10.1039/C8AN02308E



Schematic illustration of DNAzyme-based fluorescent amplified biosensor for Cys detection