

DESIGN OF A FLUORESCENCE ENHANCED APTASENSOR FOR SENSITIVE DETECTION OF SILVER IONS**

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A highly sensitive fluorescence-enhanced aptasensor was designed to detect silver ions (Ag^+) using metal-enhanced fluorescence. Interaction of Ag^+ with cytosine nucleobases was used to achieve a low detection limit. Aptamer-modified gold nanoparticles (Au NPs) were mixed with 6-carboxyfluorescein (FAM)-labeled DNA to prepare the sensor. In a solution without Ag^+ , aptamer and FAM-labeled DNA strands remained free because of repulsion between cytosine. In the presence of Ag^+ , pairs of aptamer and FAM-labeled DNA strands formed double helices through cytosine– Ag^+ –cytosine interactions. These interactions brought FAM close to the Au NPs. The number of adenine repeats in the aptamer was altered to adjust the distance between the Au NPs and FAM, and provide controllable localized surface plasmon resonance. With the optimum number of adenine repeats ($n = 24$), the linear range for detection of Ag^+ was 0.694 to 6.94 nmol/L and the detection limit was 0.694 nmol/L. The aptasensor showed excellent specificity and gave a strong detection signal for Ag^+ present at trace concentrations to overcome issues associated with the detection of weak signals.

Keywords: metal-enhanced fluorescence, aptasensor, silver ion, cytosine– Ag^+ –cytosine.

АПТАСЕНСОР НА ОСНОВЕ УСИЛЕННОЙ ФЛЮОРЕСЦЕНЦИИ ДЛЯ ЧУВСТВИТЕЛЬНОГО ДЕТЕКТИРОВАНИЯ ИОНОВ СЕРЕБРА

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Разработан высокочувствительный аптасенсор на основе усиленной флуоресценции металла для обнаружения ионов серебра Ag^+ . Взаимодействие Ag^+ с азотистыми основаниями цитозина использовано для достижения низкого предела обнаружения. Модифицированные аптамером наночастицы золота (НЧ Au) смешивали с ДНК, меченной 6-карбо-ксифлуоресцеином (FAM), для приготовления сенсора. В растворе без Ag^+ аптамер и меченные FAM нити ДНК оставались свободными. В присутствии Ag^+ пары нитей ДНК, меченных аптамером и FAM, образовывали двойные спирали за счет взаимодействий цитозин– Ag^+ –цитозин, которые привели к уменьшению расстояния между FAM и НЧ Au. Количество адениновых повторов в аптамере изменялось с целью регулирования расстояния между НЧ Au и FAM и обеспечения контролируемого локализованного поверхностного плазмонного резонанса. При оптимальном количестве адениновых повторов ($n = 24$) линейный диапазон обнаружения Ag^+ 0.694–6.94 нмоль/л, предел обнаружения 0.694 нмоль/л. Аптасенсор показал превосходную специфичность и позволил обнаружить ионы серебра, присутствующего в следовых концентрациях.

Ключевые слова: усиленная флуоресценция металла, аптасенсор, ион серебра, цитозин– Ag^+ –цитозин.

Introduction. Industrial development has led to the discharge of many metal ions into soil and water along with wastewater [1, 2]. These ions are absorbed by plants and can enter the human body through the food chain. Food safety incidents such as consumption of cadmium-contaminated rice and metal-

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contaminated vegetables have occurred. Trace metal ions accumulate in the human body, eventually causing chronic poisoning – for example, when the silver ion (Ag^+) concentration reaches a certain level it will denature proteins and various enzymes, which causes edema, and in serious cases, death [3, 4]. The World Health Organization and various countries have established limits for the residual concentrations of Ag^+ in drinking water and sewage [5]. Monitoring of metal ions to ensure they are within relevant limits is necessary for food safety and environmental protection.

The main trace metal ion detection methods currently used are atomic absorption spectroscopy, atomic fluorescence spectrometry, inductively coupled plasma mass spectrometry, mass spectrometry, and laboratory analysis [6]. Although these methods have high detection accuracies, the instruments they require are expensive and the methods are time-consuming. Consequently, many researchers have studied a variety of metal ion sensors, hoping to develop a small, portable, and cheap metal ion detector.

Fluorescence spectroscopy is widely used to detect heavy metal ions because it has high sensitivity and provides rapid responses [7]. Fluorescence sensors used for metal ion detection can be divided into those using fluorescence resonance energy transfer [8–10], photoinduced electron transfer [11, 12], intramolecular charge transfer [13, 14], metal–ligand charge transfer [15–17], chelating-enhanced fluorescence (CHEF) [18], and metal-enhanced fluorescence (MEF) [19–22]. The specificity and sensitivity are important for evaluating the advantages and disadvantages of these detection methods [23]. The specificity for a metal ion mainly depends on the group used in the sensor to capture the target. The groups used to capture metal ions are either chemical, such as various ligands and complexes, or biological, such as DNA and antibodies [24]. The ligands used for metal ion capture, such as 4-amino-3,5-bis(2-hydroxy-phenyl)-1,2,4-triazole $\text{NH}_2\text{C}_2\text{N}_3(\text{C}_6\text{H}_4\text{OH})_2$ [25], have complex structures with high dissociation constants and can be toxic. Biological groups are better than chemical groups in terms of food safety because they are nontoxic. The preparation of metal-specific monoclonal antibodies, such as tobacco mosaic virus-coated cysteine mutants [26] and tryptophan [27], involves immunodetection, which is generally expensive. In recent years, metal ion–base interactions have been studied extensively, and specific interactions such as thymine– Hg^{2+} –thymine [28] and cytosine– Ag^+ –cytosine have been found [12]. A variety of aptamers for detecting metal ions have been developed using these interactions [29]. With the available fluorescence detection methods, the detection sensitivity for a metal ion is mainly dependent on the luminous efficiency of the fluorescent group. CHEF and MEF can effectively improve the sensitivity of metal ion detection by enhancing luminous efficiency. However, CHEF generally involves the use of a complex organic molecule that could be toxic, and this method is not suitable for metal ion detection in food safety and health fields. MEF uses DNA, silica, and other nontoxic substances as an isolation layer between the metal and fluorescent groups, which effectively enhances the fluorescence signal for the highly sensitive detection of metal ions. Aptamers can be designed to capture target ions and act as isolators in MEF. These aptamers could be used to develop a fluorescent aptasensor with the high specificity of an aptamer and the high signal intensity of MEF. An aptasensor with these properties could be used to realize highly sensitive detection of metal ions using nontoxic materials for practical applications in the food safety field.

Functionalized aptamers containing multiple adenine nucleobases were prepared as an isolation layer between gold nanoparticles (Au NPs) and a fluorescent group (6-carboxyfluorescein [FAM]). The MEF could be regulated by changing the number of adenine repeats. The fluorescence aptasensor was applied to the quantitative detection of Ag^+ .

Materials and methods. The aptamers were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China). Au NPs ($\varnothing 40$ nm) were purchased from Beijing Thunder Science and Technology. Tris(2-carboxyethyl)phosphine Buffer (10 mmol/L) contained 20 mmol/L tris- CH_3COOH . Ultrapure water (18.2 $\text{M}\Omega\cdot\text{cm}$) was used in the experiments. All reagents were of analytical grade.

D1 (A_0 -aptamer): 5'-SH-CTA CCC TAG C-3'

D2 (A_{12} -aptamer): 5'-SH- A_{12} CTA CCC TAG C-3'

D3 (A_{18} -aptamer): 5'-SH- A_{18} CTA CCC TAG C-3'

D4 (A_{24} -aptamer): 5'-SH- A_{24} CTA CCC TAG C-3'

D5 (A_{30} -aptamer): 5'-SH- A_{30} CTA CCC TAG C-3'

D6 (FAM-HDNA): 5'-GCT ACC CTA G-FAM-3'

An HZQ-F200 oscillating incubator (Beijing, China) was used for hybridization. A GL-16 II centrifuge (Shanghai, China) was used for separation of samples. Fluorescence was measured using a F-7000 fluores-

cence spectrometer (Hitachi, Japan). A magnetic mixer (Hangzhou Instrument and Motor Co. Ltd.) was used for mixing the solutions. A balance (Mettler Toledo Instruments Co.) was used to weigh the samples. A working solution was prepared of AuNPs-polyA_n-aptamer mixed with FAM-HDNA. First, the aptamer was centrifuged at 7000 rpm for 1 min. Next, 10 mmol/L tris(2-carboxyethyl)phosphine solution was added to give an aptamer (D1–D6) concentration of 5 mol/L. Au NPs (0.05 mg/mL) were then mixed with the aptamers (D1–D5) in a set ratio, and the mixture was incubated at 37°C for 12 h. NaCl (2 mol/L) was added twice at 3 h intervals to give a final NaCl concentration of 0.1 mol/L. The supernatant was removed by centrifugation at 14 000 rpm for at least 20 min. Finally, 600 µL of tris-CH₃COOH and 20 µL of 5 mol/L FAM-HDNA were added, and the samples were stored at 4°C for later use. Aliquots of a solution of Ag⁺ (100 µL) at different concentrations were added to the working solution and the samples were incubated at 37°C for 1 h. A solution of Ag⁺ (600 µL) was also placed in a culture plate. The fluorescence intensity of the working solution was measured using a fluorescence spectrometer, and the relationship between the Ag⁺ concentration and fluorescence intensity was calculated.

Results and discussion. *Detection principle of the proposed method and its feasibility.* The detection principle of the aptasensor for Ag⁺ is shown in Fig. 1. First, polyA_n-aptamer attached to the Au NPs surface via Au–S bonds. FAM-HDNA was then added, and the polyA_n-aptamer and FAM-HDNA DNA strands were in a free state (structure A in Fig. 1). The fluorescence intensity of the working solution at this stage was relatively low. Next, Ag⁺ was added and pairs of polyA_n-aptamer and FAM-HDNA DNA strands formed double helices through cytosine–Ag⁺–cytosine interactions. This brought FAM-HDNA close to the Au NPs to form AuNPs-polyA_n-FAM nanostructure (structure B in Fig. 1). The formation of this structure effectively amplified the fluorescence signal of FAM through MEF. As the Ag⁺ concentration increased, the number of cytosine–Ag⁺–cytosine interactions and the intensity of the fluorescence signal also increased. The Ag⁺ concentration could be determined from the degree of enhancement of the fluorescence signal. Compared with the use of FAM on its own, the sensor gave a stronger output signal. Therefore, this method will be useful for the detection of Ag⁺ at trace concentrations and will overcome issues associated with the detection of weak signals.

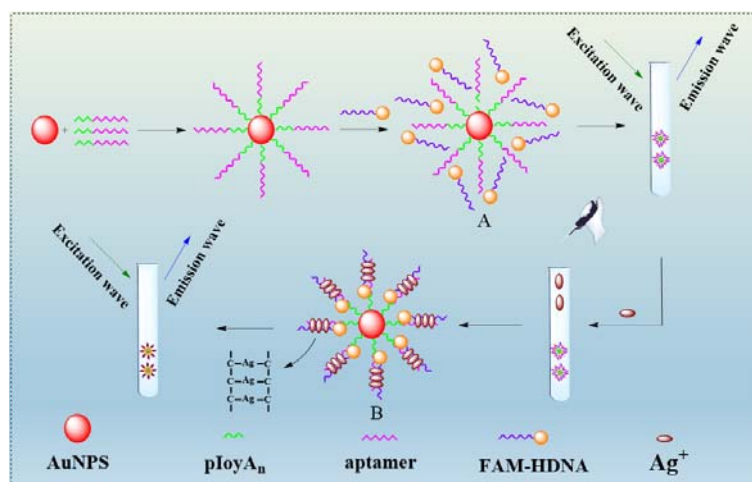


Fig. 1. Schematic diagram of the detection of silver ions by the aptasensor.

To verify the feasibility of application of the sensor, the fluorescence intensities of FAM-HDNA, D3/FAM-HDNA, Au/D3/FAM-HDNA, and Au/D3/FAM-HDNA/Ag⁺ were measured. Au/FAM-HDNA had the lowest fluorescence intensity (Fig. 2) because some of the fluorescence of FAM was quenched by the Au NPs. The fluorescence intensity of the Au/FAM-HDNA solution was lower than that of FAM-HDNA. The fluorescence intensities of D3/FAM-HDNA and FAM-HDNA were similar and both were higher than that of Au/FAM-HDNA. Without AuNPs in the solution, the DNA strands of D3 and FAM-HDNA were in a free state. The fluorescence intensity of Au/D3/FAM-HDNA was higher than the fluorescence intensities of FAM-HDNA and D3/FAM-HDNA. In these solutions, the adenine repeats around the Au NPs formed a protective layer, which prevented fluorescence quenching of FAM and enhanced the fluorescence of FAM-HDNA. With addition of Ag⁺, the concentration of Ag⁺ was determined using the degree of fluorescence enhancement. Our results confirmed that the sensor could be used to detect Ag⁺.

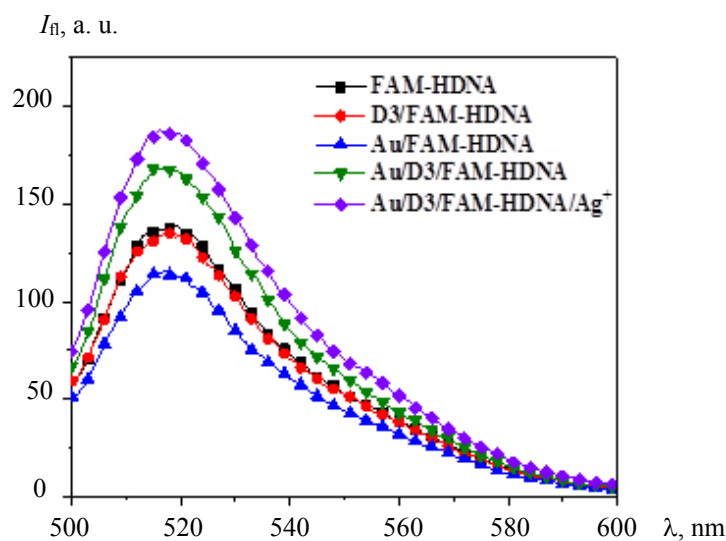


Fig. 2. Detection of silver ions by the aptasensor.

Optimization of the experimental conditions. The MEF effect is closely related to the distance between the Au NPs and the fluorophore [21]. To obtain a strong fluorescence signal, we optimized the distance between the Au NPs and the fluorophore. The distance was adjusted by changing the number of adenine repeats with the knowledge that three adenine repeats cover a distance of 1 nm [30]. We measured the fluorescence intensities of solutions containing 10 nmol/L Ag^+ and aptamers with 0, 12, 18, 24, and 30 adenine repeats. The highest fluorescence intensity was obtained with 24 adenine repeats, followed by 18, and then 30 (Fig. 3). The solutions with distances of 0 and 4 nm had low fluorescence intensities. When the distance between the Au NPs and the fluorophore is between 0 and 5 nm, fluorescence resonance energy transfer will occur, resulting in fluorescence quenching [19] and a decrease in the fluorescence intensity. If the Au NPs and the fluorophore are too far apart, they will not affect each other and the fluorescence intensity will be equivalent to that of the fluorophore alone [21]. Our results showed that a distance of 8 nm between the Au NPs and FAM was optimum for MEF. Distances of 6 nm and 10 nm also provided some MEF, but the effect was not as obvious as that with a distance of 8 nm. Therefore, the polyA_n-aptamer was selected for subsequent experiments.

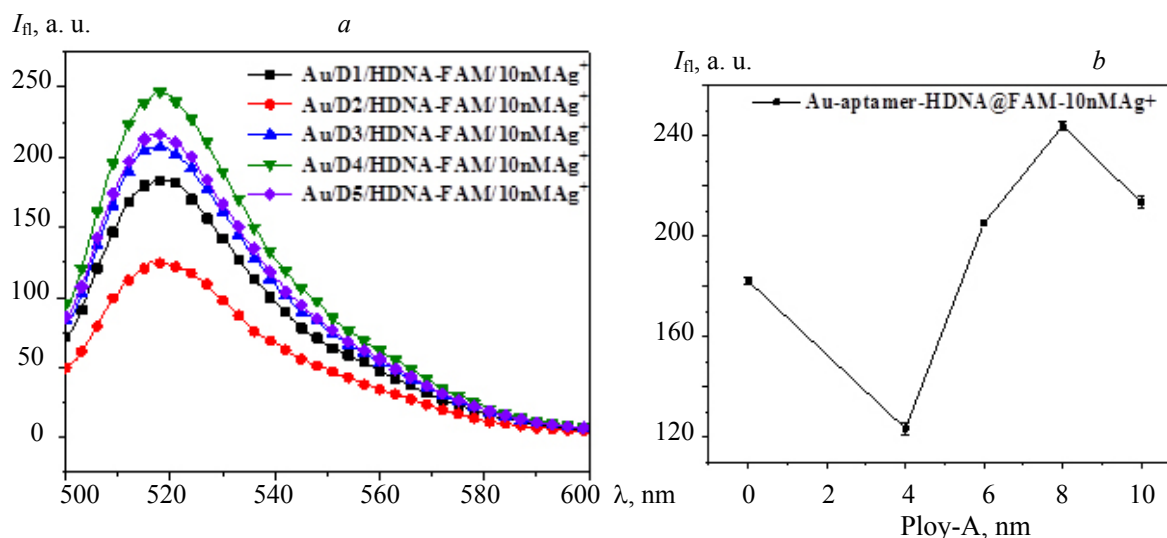


Fig. 3. Optimization of the thickness of the isolation layer: (a) fluorescence spectra of Au/polyA_n-aptamer/FAM-HDNA/Ag⁺ solutions with different isolation layer thicknesses; (b) relationship between the fluorescence intensity of Au/polyA_n-aptamer/FAM-HDNA/Ag⁺ and the isolation layer thickness. The concentration of was Ag^+ 10 nmol/L.

To improve the sensitivity of the sensor, we optimized the ratio of AuNPs to aptamers [31]. If there are too many aptamers on the surface of the Au NPs, some complementary chains will not be able to access the aptamers in order to bind. By contrast, if there are too few aptamers on the Au NPs, the aptamers may be located close to the surface of Au NPs, and the fluorescence of FAM will be quenched by the Au NPs [32]. To investigate the ratio between the Au NPs and the aptamer, the concentration of aptamer was fixed at 5 mol/L and the volume at 20 μL , and the volume of the 0.05 mg/mL Au NPs solution was changed. The fluorescence intensity of the working solution was measured for Au NPs/aptamer volume ratios of 0.25:1, 0.5:1, 1.25:1, 2:1, 2.5:1, and 4:1. When the volume ratio was between 0.25:1 and 2:1, the fluorescence intensity of the working solution gradually increased with increases in the volume ratio (Fig. 4). This occurred because the increase in aptamers on the surface of the Au NPs resulted in arrangement of the aptamers in a linear fashion rather than coating with Au NPs. When the volume ratio was between 2:1 and 4:1, the fluorescence intensity decreased with increases in the volume ratio. This occurred because as the density of aptamers on the surface of the Au NPs increased, it was difficult for complementary chains to access the aptamers for binding, which resulted in a decrease in the fluorescence intensity. Therefore, to improve the sensitivity of the sensor, an Au NPs/aptamer volume ratio of 1:0.5 was selected for subsequent experiments.

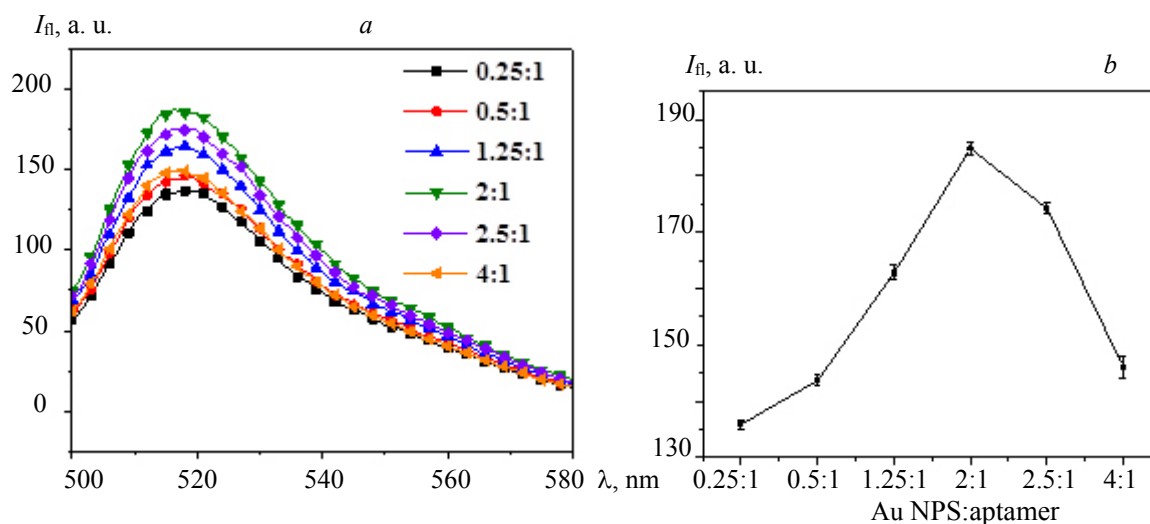


Fig. 4. Optimization of the Au NPs to aptamer volume ratio: (a) fluorescence spectra of Au NPs and aptamers with different volume ratios; (b) relationship between the volume ratio of AuNPs/aptamer and the fluorescence intensity.

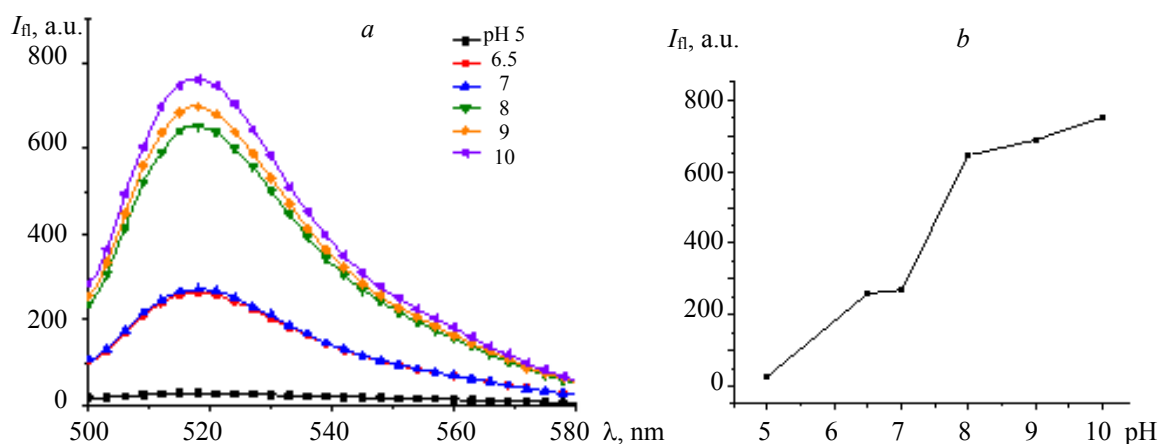


Fig. 5. Optimization of the pH: (a) fluorescence spectra of the working solution at different pH values; (b) relationship between different pH values and the fluorescence intensity.

Optimization of the pH is crucial to improve the sensitivity of a sensor [33]. Low pH will affect the fluorescence intensity of the working solution [34], while high pH will prevent cytosine–Ag⁺–cytosine interactions. To observe the effect of pH values between 5 and 10 on the fluorescence intensity of the working solution, a tris-CH₃COOH buffer with different pH values was added to the working solution. In the pH range of 5–10, the fluorescence intensity of the working solution increased at higher pH values (Fig. 5). A low pH could inhibit the activity of FAM, and reduce the fluorescence signal. A high pH could enhance the fluorescence signal, but Ag⁺ can react with OH[−] under alkaline conditions and this might make cytosine–Ag⁺–cytosine interactions difficult. Therefore, to improve the intensity of the fluorescence signal and reduce interference of OH[−] in Ag⁺ detection, we selected a buffer solution pH 7.0.

Detection of Ag⁺ at different concentrations. To evaluate the detection range of the sensor, a working solution was prepared containing 24 cytosine nucleobases. The volume ratio of Au NPs to aptamer was 1:0.5, and the pH was 7. The fluorescence intensity of the working solution was detected after adding Ag⁺ at 5, 10, 20, 30, and 50 nmol/L. The fluorescence intensity increased as the Ag⁺ concentration increased (Fig. 6a). This occurred because there was more cytosine–Ag⁺–cytosine interactions, and more FAM moved close to the Au NPs. The linear regression equation between different concentrations of Ag⁺ and the fluorescence intensity was $y = 0.007x + 151$, where y is the fluorescence intensity and x is the Ag⁺ concentration (Fig. 6b), and the R^2 was 0.99. The detection range of the sensor was 0.694–6.94 nmol/L and the detection limit was 0.694 nmol/L. Compared with colorimetric, electrochemical, and similar methods in the literature (Table 1), the detection limit of the aptasensor prepared in this study is relatively low.

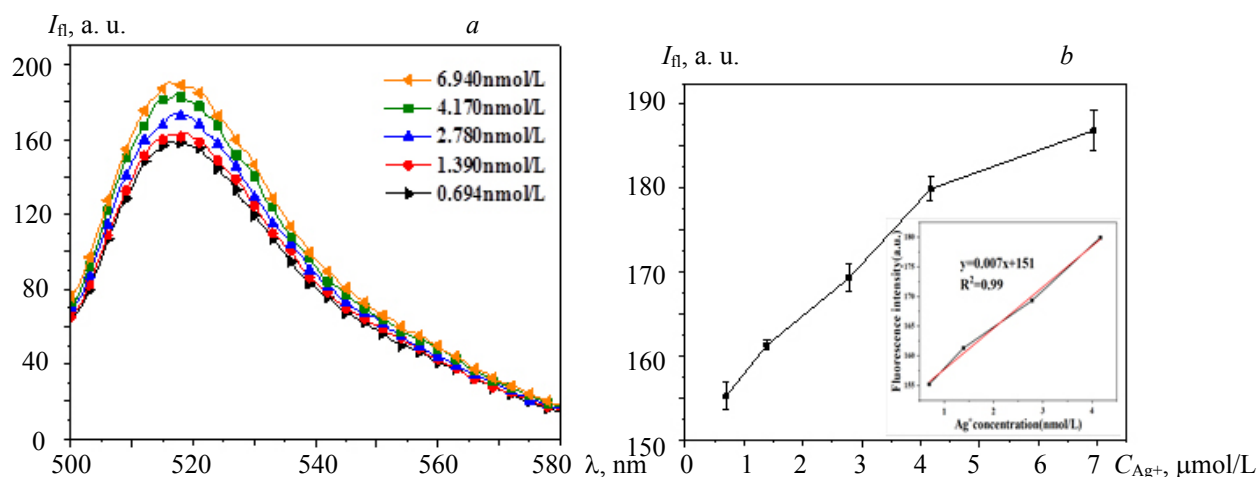


Fig. 6. Detection of Ag⁺: (a) fluorescence spectra with different concentrations of Ag⁺ in the working solution; (b) relationship between different concentrations of Ag⁺ and the fluorescence intensity.

TABLE 1. Comparison of Different Sensors for Ag⁺ Detection

Method	Linear range	LOD	Reference
Colorimetric	0–100 μM	26 nM	[35]
	0.05–0.9 μM	20 nM	[36]
Electrochemistry	10–500 nM	1.3 nM	[37]
	2–100 nM	1.5 nM	[38]
Fluorescence	0–90 mM	320 nM	[39]
	50–500 nM	10 nM	[40]
	10–500 nM	10 nM	[41]
	0.5–13 μM	0.08 μM	[42]
	0–45 μM	15 nM	[43]
	3–30 μM	0.26 μM	[44]
	5–60 μM	0.226 μM	[45]
	0.1–1.5 μM	68 nM	[46]
	0.694–6.94 nM	0.694 nM	This work

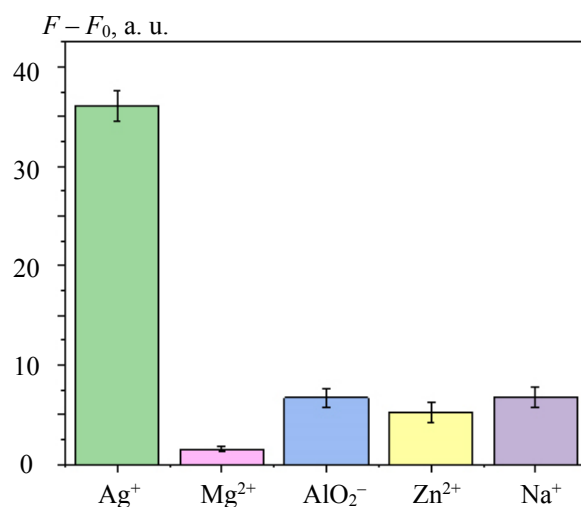


Fig. 7. Changes in the fluorescence intensity of the sensor after addition of different ions at 6.94 nmol/L.

Specificity is an important parameter used to evaluate the performance of a sensor. To determine the specificity of the sensor, the fluorescence intensity of a working solution containing 6.94 nmol/L Mg^{2+} , K^+ , Al^{3+} , Zn^{2+} , Fe^{2+} , and Na^+ was analyzed under the optimum experimental parameters. The value of $F - F_0$ was used to calculate the change in the fluorescence intensity after ion addition, where F and F_0 are the fluorescence intensity of the working solution and the fluorescence intensity after ion addition, respectively. Addition of Ag^+ resulted in a large change in the fluorescence intensity (Fig. 7). Only slight changes in the fluorescence intensity were observed with the other ions, which indicated that the fluorescence-enhanced aptasensor had excellent specificity for Ag^+ .

Conclusions. We developed a fluorescent aptamer sensor with high output intensity using metal-enhanced fluorescence. To realize the metal-enhanced fluorescence effect and improve the detection limit of the sensor, we optimized the preparation of the sensor and applied it to detection of different concentrations of Ag^+ . When the number of adenine repeats was 24, the Au NPs to aptamer ratio was 1:0.5, and the pH was 7, the detection limit was 694 pmol/L and the detection range was 0.694–6.94 nmol/L. The sensor was specific to Ag^+ and did not respond to other metal ions. This sensor could be applied to other metal ions after modification of the aptamer.

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