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A sensitive fluorometric sensor for Ag^+ based on the hybridization chain reaction coupled with a glucose oxidase dual-signal amplification strategy†

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In this work, an efficient and sensitive fluorometric sensor was developed to detect silver ions (Ag^+). It is based on the cytosine- Ag^+ -cytosine (C- Ag^+ -C) structure *via* a dual-signal amplification strategy using glucose oxidase (GOx) and the hybridization chain reaction (HCR). A silver-coated glass slide (SCGS) acts as an ideal material for separation. Cytosine rich (C-rich) capture DNA (C-DNA) assembled themselves on the SCGS *via* Ag-S bonds and hybridized with signal DNA (S-DNA) to trigger the HCR. With specific base-pairing, the S-DNA and HCR products bind on the SCGS. Then, the GOx-biotin-streptavidin (SA) complexes bind to the HCR products through SA-biotin interactions. Owing to the formation of a particular C- Ag^+ -C structure between two neighboring C-rich C-DNA on the SCGS, the C-DNA/S-DNA/HP1-GOx/HP2-GOx complex gradually moved away from the SCGS as the concentration of Ag^+ increased and the combined GOx fell into the buffer. H_2O_2 could be generated during the oxidation of glucose, catalyzed by GOx in the buffer. Afterward, H_2O_2 could oxidize the substrate (3-(*p*-hydroxyphenyl)-propanoic acid) when Horseradish peroxidase was present, giving rise to blue fluorescence. The proposed strategy reached a limit of detection (LOD) of 1.8 pmol L^{-1} with a linear detection range of 5 to 1000 pmol L^{-1} for Ag^+ . Moreover, this assay has been commendably used for the detection of Ag^+ in actual samples with fairly good results.

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1 Introduction

Environmental pollution and human health problems caused by heavy metal ions have aroused extensive attention.^{1,2} The silver ion (Ag^+) is a particularly notorious heavy metal ion because it is highly poisonous and widespread in water, soil, atmosphere, crops, and fishery products.³⁻⁶ Ag^+ can inactivate various metabolites and sulfhydryl enzymes by interacting with thiol, amino, and carboxyl groups to produce cytopathogenic effects.^{7,8} Taking these adverse factors into account, it is urgent and necessary to develop a highly sensitive and selective assay for Ag^+ monitoring. Currently, many strategies have been established for the detection of Ag^+ , including atomic absorption spectroscopy,^{9,10} inductively coupled plasma mass spectroscopy,^{11,12} and electrochemical analysis.^{13,14} However, traditional atomic absorption spectroscopy cannot be used for the determination of trace content samples and it has a narrow linear range, which is inconvenient for practical applications. Besides, inductively coupled plasma mass spectrometry requires costly instruments

and a well-trained analyst, and is sensitive to the sample medium and temperature. Although the accuracy and sensitivity of electrochemical analysis methods are high, they are susceptible to a variety of interference currents and have poor selectivity in complex samples. Methods based on real-time fluorescence PCR have both pros and cons. Despite their high sensitivity, they have shortcomings including being prone to false-positive signals and requiring expensive equipment and complicated operating procedures. Since Ono *et al.* reported that Ag^+ specifically interacts with cytosine (C) to form a stable C- Ag^+ -C structure,^{15,16} several C- Ag^+ -C based sensors for Ag^+ analysis have been reported, such as fluorescent, colorimetric, and electrochemical sensors.¹⁷⁻¹⁹ Moreover, the peculiar C- Ag^+ -C interactions guarantee prominent selectivity because C-C mismatches bind to Ag^+ beyond everything else. Due to the limitations of the above methods and the specific C- Ag^+ -C structure, this study proposes a fluorescence assay for Ag^+ based on the C- Ag^+ -C structure, which has many advantages including simplicity, high sensitivity, high selectivity, and easy operation.

Up to now, to advance the determination sensitivity of sensors, methods based on signal amplification strategies have been extensively investigated, comprising polymerase chain reaction (PCR),^{20,21} hybridization chain reaction (HCR),^{22,23} rolling circle amplification (RCA)^{24,25} and strand displacement amplification

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(SDA).^{26,27} Among these strategies, HCR is regarded as a promising tool for signal amplification by virtue of its enzyme-free nature, simplicity, economy, isothermality, and excellent sensitivity. Moreover, HCR has been extensively used for the determination of oligonucleotides,²⁸ proteins,²⁹ and tumor cells.³⁰

Oligonucleotides and some biomolecules can be immobilized on solid substrates to isolate bound biomolecules from free molecules. This strategy has been successful in the study of surface functionalization on glass, nano-magnetic microspheres, nanoparticles, electrodes, and so forth.^{31–35} Nevertheless, these immobilized means using in the above researches still exist downsides like costly, inconvenient, inefficient and so on. This paper presents a preferable separation material, namely, silver-coated glass slide (SCGS),^{36–39} which was readily obtained by the silver mirror reaction.^{40–42} In addition, thiolated DNA can assemble themselves on the surface of silver through covalent forces using Ag–S bonds.^{43–45}

GOx, an inexpensive and stable aerobic dehydrogenase, serves as a signal amplifier for the fabrication of sensors to detect glucose, proteins, nucleic acid, and biological molecules.^{46–49} Inspired by the aforementioned work, herein, based on C–Ag⁺–C interactions, we present an efficient and sensitive fluorometric sensor for the detection of Ag⁺ using HCR/GOx dual signal amplification and SCGS. This paper may offer a novel way for the determination of Ag⁺ in the future.

2 Experimental section

2.1 Reagents and chemicals

Horseradish peroxidase (HRP), bovine serum albumin powder (BSA), GOx, streptavidin (SA), and all of the synthetic oligonucleotides (Table S1†) used in this work were obtained commercially from Sangon Bioengineering Technology and Services Co. Ltd (Shanghai, China). 3-(*p*-Hydroxyphenyl)-propanoic acid (HPPA), trichloromethyl phosphate (TCEP), argentum nitricum, glucose, 1% ammonia spirit, and the inorganic reagents were obtained from Sinopharm Chemical Reagent Co. Ltd (Beijing, China). The human sera were obtained from Hongquan biological technology Co., Ltd (Guangzhou, China). Ultra-pure water (18.1 MΩ cm, at 25 °C) was utilized in all of the experiments. All of the reagents used in the experiments were analytically pure. All of the oligonucleotides were dissolved in phosphate-buffered saline (PBS, pH 7.4, 20 mmol L^{−1}, 50 mmol L^{−1} NaNO₃). The 5% BSA solution was prepared by dissolving 5.0 g bovine serum albumin powder in 100 mL PBS buffer solution. All of the working solutions listed above were kept in a fridge at 4 °C until use.

The method of preparing SCGS^{36,37} and GOx–biotin^{38,39} is outlined by Li *et al.* Hairpin probes, HP1 and HP2, were annealed in a water bath at 95 °C for 5 min, which was followed by slowly cooling to the indoor temperature to form the stable stem-loop structure before use.

2.2 Apparatus

A PHS-3E pH meter (Lei-ci Instrument Factory, Shanghai, China) was used for pH measurements. The AAS measurements

were performed using a Z-2000 series polarized Zeeman atomic absorption spectrometer (Hitachi, Japan). The fluorescence spectra were acquired using an F-4600 Hitachi spectrofluorimeter (Japan) equipped with excitation at 320 nm and the emission spectra were obtained from 360 to 460 nm. The excitation and emission slits were 5 nm. The fluorescence intensity of the oxidation products of HPPA at 400 nm was utilized to appraise the performance of the presented method.

2.3 Fabrication of the sensor

Firstly, according to a reported method, the captured DNA (C-DNA) was immobilized onto a silver-coated glass slide by Ag–S bonds.^{40,41} Then, the sample was soaked in 5% BSA buffer solution for 15 minutes to plug any potential residual active sites and prevent non-specific adsorption. Then, the C-DNA immobilized SCGS was immersed in a 100 nmol L^{−1} signal DNA (S-DNA) solution for 1 hour at 25 °C. After that, the C-DNA/S-DNA modified SCGS and 400 nmol L^{−1} HP1 and HP2 were mixed in a buffer solution for 80 min at 37 °C to initiate the HCR reaction and form long-nicked double helices. After the addition of 2 μg mL^{−1} SA and 50 μg mL^{−1} GOx–biotin, the C-DNA/S-DNA/HP1-GOx/HP2-GOx complex immobilized SCGSs were submerged in buffer solutions of different Ag⁺ concentrations for 1 h at 37 °C. Afterward, the aforementioned solution was mixed with 50 mmol L^{−1} glucose and reacted at 37 °C for 1 h to generate H₂O₂. Ultimately, 0.5 μg mL^{−1} HRP and 50 μmol L^{−1} HPPA were added to the aforementioned solution and incubated at 37 °C for 30 min in the dark, and the eventual solution was investigated *via* fluorescence spectrophotometry.

2.4 Real sample application

The concentration of Ag⁺ in Ruiyun Lake water from Zhanjiang city and tap water were monitored by fluorescence spectrophotometry. The water samples were filtered first using a 10 μm filter and then using a 0.45 μm filter to facilitate the removal of debris and impurities. In addition, the determination of Ag⁺ in biological samples is crucial for monitoring health. Hence, human sera samples were also analyzed to examine the feasibility of the presented sensor. The human sera were transferred into two sterile centrifuge tubes and centrifuged at 4000 rpm for 12 min. The supernatant was collected and filtered (0.45 μm filter).

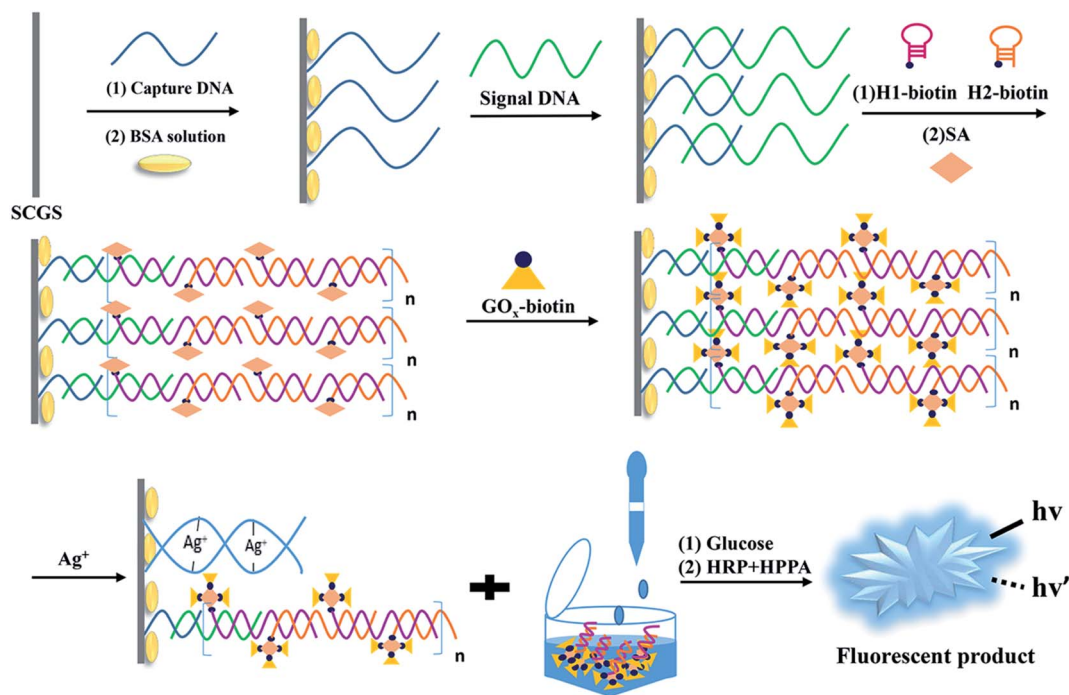
The standard addition method was adopted for quantifying Ag⁺. Ag⁺ stock solutions with concentrations ranging from 5 to 1000 pmol L^{−1} were added to the aliquots of the above-mentioned samples. Eventually, each sample was diluted 10 times with phosphate-buffered saline and then the proposed method was performed to detect Ag⁺.

3 Results and discussion

3.1 Working mechanism

Scheme 1 depicts the basic principle of the Ag⁺ assay. The hairpin sequences (HP1 and HP2) modified with biotin at the 5' end are stabilized in the absence of S-DNA. Since it can specifically bind to biotin, SA is utilized to couple HP1 and HP2 with GOx–biotin, which serves as a signal amplification component. In our





Scheme 1 Schematic of the Ag^+ detection method based on the C- Ag^+ -C structure, which utilizes a HCR/GOx dual-signal amplification strategy and SCGS as an ideal separation material.

strategy, C-rich C-DNA can assemble themselves on the surface of SCGSs *via* Ag-S bonds and hybridize with S-DNA. HCR efficiently primes the sample to form long-nicked duplex DNA (S-DNA/HP1-biotin/HP2-biotin structures) in the presence of S-DNA. GOx-biotin adheres to the HCR product *via* SA-biotin interactions. With specific base-pairing between C-DNA and S-DNA, the S-DNA/HP1-GOx/HP2-GOx complexes are attracted to the surface of the SCGSs. The SCGSs modified with the above complexes were then immersed in a buffer solution containing Ag^+ . Then, Ag^+ enters the binding site of the mismatched C-C bases to form a stable “C- Ag^+ -C” structure between two adjacent C-rich C-DNA on the SCGS and the complexes move away from the SCGS, resulting in the combined GOx falling into the buffer solution. Thus, with higher Ag^+ concentrations in the buffer solution, more GOx can be found in the buffer solution. In the presence of HRP, HPPA is oxidized by H_2O_2 , which is generated during the GOx-catalyzed oxidation of glucose and gives rise to the fluorescence signals. Therefore, the concentration of Ag^+ could be appraised from the fluorescence intensity.

3.2 Feasibility study on the proposed sensor

To explore the feasibility of this approach, the fluorescence emission spectra of the different mixtures were recorded. As displayed in Fig. 1, the fluorescence intensity is feeble without C-DNA because the C-DNA/S-DNA/HP1/HP2 structure has not formed (curve a). The same situation occurs in the absence of S-DNA and HP1. Likewise, the fluorescence spectrum displays a weak signal without SA-biotin-GOx (curve b), indicating that the fluorescent product cannot be yielded in the absence of GOx. Although the nicked double-helix polymer cannot be produced without HP2, the C-DNA/S-DNA/HP1-GOx structure can still be

formed, causing a slight increase in fluorescence (curve c). The fluorescence intensity was weak in the absence of Ag^+ (curve d). Nevertheless, as Ag^+ can enter the binding site of the mismatched C-C bases and form a stable “C- Ag^+ -C” structure between two adjacent C-rich C-DNA, the C-DNA/S-DNA/HP1-GOx/HP2-GOx complexes gradually move away from the SCGSs with increasing Ag^+ concentration. Thus, the fluorescence intensity increased significantly (curve e). The above results powerfully demonstrate the feasibility of this sensor for Ag^+ determination.

3.3 Sensitivity of the proposed sensor

Different concentrations of Ag^+ were added to investigate the sensitivity of the sensor under optimal conditions. As illustrated

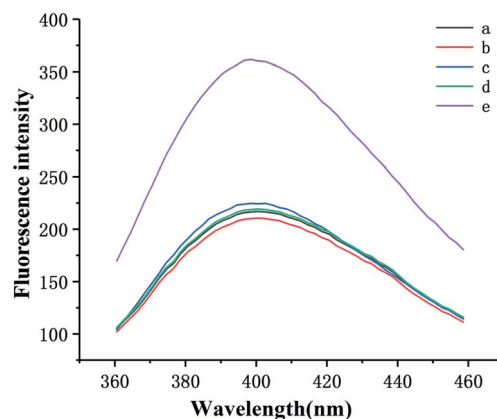


Fig. 1 Fluorescence emission spectra of the different solutions: (a) mixture without immobilized C-DNA; (b) mixture without SA-biotin-GOx; (c) mixture without HP2; (d) mixture without Ag^+ ; (e) mixture.

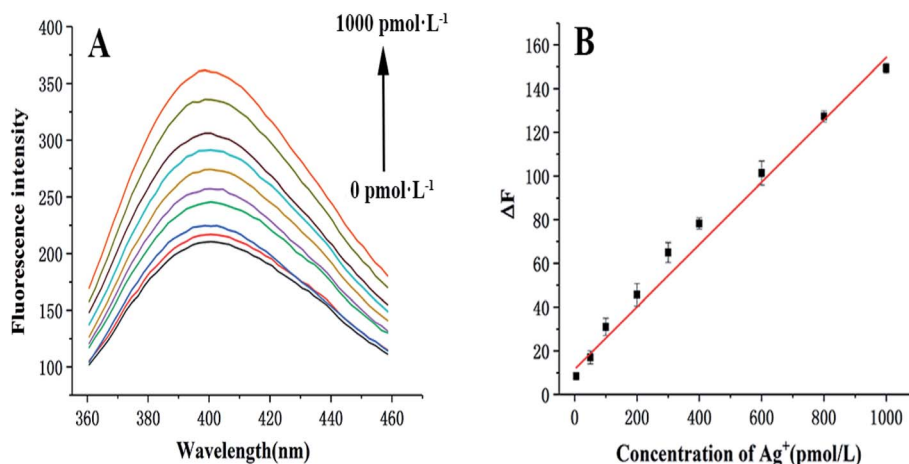


Fig. 2 Fluorescence spectra (A) and calibration plot (B) of Ag^+ . Error bars represent the standard deviation of three independent experiments. Experimental conditions: $1.0 \mu\text{mol L}^{-1}$ C-DNA, 100 nmol L^{-1} S-DNA, 400 nmol L^{-1} HP1 and HP2, $2 \mu\text{g mL}^{-1}$ SA, $50 \mu\text{g mL}^{-1}$ GOx-biotin, 50 mmol L^{-1} glucose, $0.5 \mu\text{g mL}^{-1}$ HRP and $50 \mu\text{mol L}^{-1}$ HPPA.

Table 1 Comparison of the proposed method with other reported assays

Analytical method	Linear range	LOD	Reference
Electrochemical biosensor based on DNA-Au bio-bar codes and silver	5 pmol L^{-1} to $50 \mu\text{mol L}^{-1}$	2 pmol L^{-1}	2
Colorimetric assay based on gold nanoparticle oligomers	0.5 pmol L^{-1} to 5 nmol L^{-1}	$0.246 \text{ pmol L}^{-1}$	4
Gold nanoparticle-based fluorescence polarization	$50\text{--}750 \text{ nmol L}^{-1}$	9.5 nmol L^{-1}	5
Fluorescent chemosensor based on thionine	$5\text{--}800 \text{ fmol L}^{-1}$	5.0 fmol L^{-1}	50
Room-temperature phosphorescence assay	$0.1\text{--}10 \mu\text{mol L}^{-1}$	36 nmol L^{-1}	51
Electrochemical biosensor based on magnetic nanoparticles	1 fmol L^{-1} to 10 pmol L^{-1}	0.5 fmol L^{-1}	52
Biosensor based on nanoporous gold and anionic intercalator	0.1 nmol L^{-1} to $1 \mu\text{mol L}^{-1}$	0.48 pmol L^{-1}	53
Sensor based on HCR and GOx	$5\text{--}1000 \text{ pmol L}^{-1}$	1.8 pmol L^{-1}	This work

in Fig. 2A and B, the fluorescence intensity enhanced with increasing Ag^+ concentration. It shows a positive linear concentration range between 5 pmol L^{-1} and 1000 pmol L^{-1} of Ag^+ , with a linear regression equation of $\Delta F = 0.14C + 15.51$ ($R^2 = 0.993$, C refers to the concentration of Ag^+ (pmol L^{-1}), ΔF represents the change in fluorescence intensity, assessed by the equation: $\Delta F = F_{\text{target}} - F_{\text{blank}}$, where F_{blank} represents the fluorescence signal of the mixture without Ag^+ , F_{target} denotes the fluorescence signal of the mixture in the presence of Ag^+ at different concentrations.). The LOD of this sensor is 1.8 pmol L^{-1} ($N = 3$, $\text{RSD} = 3.2\%$), which was calculated using the equation: $\text{DL} = 3\sigma/\text{slope}$ (σ represents the relative standard deviation of the blank sample and slope represents the slope of the linear regression equation).

Compared with several other assays (Table 1), the dynamic range and LOD of the proposed sensor are better than those of other relevant studies. Moreover, there are other prominent characteristics of this sensor, such as the use of SCGSs as an ideal separation material, the dual-signal amplification strategy based on HCR/GOx, and the C- Ag^+ -C structure as a recognition element.

3.4 Selectivity of the proposed sensor

The selectivity of this sensor was investigated using other environmentally related cations. A mixture of all of the cations

(including Ag^+) was tested under the same conditions. As demonstrated in Fig. 3, a prominent enhancement in fluorescence intensity is observed in the presence of Ag^+ . This outcome uncovers that this method exhibits prominent

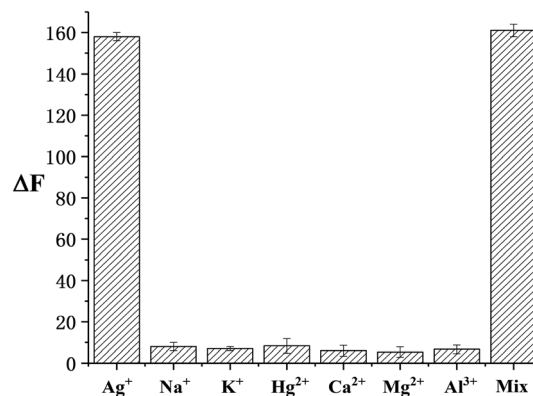


Fig. 3 Selectivity of the sensor towards Ag^+ in the presence of other metal ions. Error bars denote the standard deviation of three independent experiments ($N = 3$, $\text{RSD} = 4.5\%$). Experimental conditions: 1000 pmol L^{-1} Ag^+ , 10 nmol L^{-1} other metal ions, $1.0 \mu\text{mol L}^{-1}$ C-DNA, 100 nmol L^{-1} S-DNA, 400 nmol L^{-1} HP1 and HP2, $2 \mu\text{g mL}^{-1}$ SA, $50 \mu\text{g mL}^{-1}$ GOx-biotin, 50 mmol L^{-1} glucose, $0.5 \mu\text{g mL}^{-1}$ HRP and $50 \mu\text{mol L}^{-1}$ HPPA.



Table 2 Results of the determination of Ag⁺ in real samples (n = 3)

Sample	Added (pmol L ⁻¹)	Proposed method mean ^a ± SD ^b (pmol L ⁻¹)	AAS mean ^a ± SD ^b (pmol L ⁻¹)
Ruiyun Lake water	5.0	5.3 ± 0.3	5.1 ± 0.4
Ruiyun Lake water	50.0	52.1 ± 2.6	53.7 ± 3.1
Ruiyun Lake water	500.0	513.7 ± 29.0	487.3 ± 34.7
Ruiyun Lake water	1000.0	969.7 ± 39.0	1063.1 ± 38.4
Tap water	5.0	5.4 ± 0.3	5.7 ± 0.2
Tap water	50.0	55.2 ± 2.7	49.7 ± 3.7
Tap water	500.0	488.4 ± 22.1	491 ± 28.3
Tap water	1000.0	979.1 ± 36.4	1012.7 ± 38.9
Human sera	5.0	5.2 ± 0.3	5.3 ± 0.3
Human sera	50.0	47.3 ± 3.4	50.7 ± 4.1
Human sera	500.0	488.9 ± 26.1	488.3 ± 27.6
Human sera	1000.0	971.2 ± 33.7	983.7 ± 46.8

^a Mean of three determinations. ^b SD, standard deviation.

selectivity towards Ag⁺ monitoring despite high concentrations of other cations.

3.5 Real sample detection

The extensive range of applicability of the presented assay was assessed by recovery studies using lake water, tap water, and human sera. The results were then compared with those obtained through atomic absorption spectrometry (AAS), which served as a reference standard. The results of the experiments are presented in Table 2. The high recovery and satisfactory RSD values proved that this sensor is both practical and reliable for the determination of Ag⁺ in authentic samples.

4 Conclusion

In this study, an efficient and sensitive fluorescence method to determine Ag⁺ was established based on a C-Ag⁺-C structure and GOx/HCR dual-signal amplification using SCGs as the separation material. This approach has some prominent characteristics. First, the proposed approach has been successfully used to detect Ag⁺ in complicated systems with many components including biological media. Further, the LOD of this assay is low because of the dual-signal amplification strategy based on the HCR and GOx. Moreover, this assay has been commendably used for the detection of Ag⁺ in actual samples with fairly good results. We firmly believe that this assay may provide a novel way for the determination of Ag⁺ in the future.

Live subject statement

All of the experiments in this study were performed in compliance with the guidelines of the ethics committee of Guangdong Ocean University, China. It was approved by the ethical committee of Guangdong Ocean University. Informed consent was obtained from the human subjects prior to obtaining the plasma sera.

Conflicts of interest

There are no conflicts of interest to declare.

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