Anal. Chem. **2008,** *80,* 7206–7212

Articles

Ultrasensitive Flow Injection Chemiluminescence Detection of DNA Hybridization Using Signal DNA Probe Modified with Au and CuS Nanoparticles

Shusheng Zhang,* Hua Zhong, and Caifeng Ding*

Key Laboratory of Eco-chemical Engineering, Ministry of Education. College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, China

A novel and sensitive flow injection chemiluminescence assay for sequence-specific DNA detection based on signal amplification with nanoparticles (NPs) is reported in the present work. The "sandwich-type" DNA biosensor was fabricated with the thiol-functionalized capture DNA first immobilized on an Au electrode and hybridized with one end of target DNA, the other end of which was recognized with a signal DNA probe labeled with CuS NPs and Au NPs on the 3′-and 5′-terminus, respectively. The hybridization events were monitored by the CL intensity of luminol−H_2O_2−Cu^{2+} after the cupric ions were dissolved from the hybrids. We demonstrated that the incorporation of Au NPs in this sensor design significantly enhanced the sensitivity and the selectivity because a single Au NP can be loaded with hundreds of signal DNA probe strands, which were modified with CuS NPs. The ratios of Au NPs, signal DNA probes, and CuS NPs modified on the gold electrode were ~1/101/103. A preconcentration process of cupric ions performed by anodic stripping voltammetry technology further increased the sensor performance. As a result of these two combined effects, this DNA sensor could detect as low as femtomolar target DNA and exhibited excellent selectivity against two-base mismatched DNA. Under the optimum conditions, the CL intensity was increased with the increase of the concentration of target DNA in the range of 2.0×10^{-14}–2.0×10^{-12} M. A detection limit of 4.8×10^{-15} M target DNA was achieved.

Sequence-specific DNA detection associated with gene analysis, tissue matching, pathogenic diseases, and forensic applications has become increasingly important in molecular diagnostics.[1−3] Detection sensitivity of the DNA sensor is usually determined by signal variation amplitude of the hybridization event. As a result, various technologies such as surface plasmon resonance,[4] laser diffraction,[5] surface-enhanced Raman spectroscopy,[6,7] and array-based electrical detection[8] have been proposed to increase the signal turnovers in the corresponding DNA hybridization. Amplified transduction of DNA sensing events was accomplished by Au NPs with Ag amplification.[5,8−11] Different electrochemical and optical DNA sensors for the amplified analysis of DNA have been developed owing to their high sensitivity and compatibility.[12−14]

In recent years, metal or semiconductor nanoparticles (NPs) with unique optical and electrical properties[15−19] have also been widely employed as labels for the amplified detection of DNA to overcome the safety problems, poor sensitivity, and poor stability associated with the radioisotopic, fluorescent, and enzyme labels. Colloidal Au NPs, which can be prepared easily, have been widely used for the analysis of DNA.[9−11,20−23] Willner's group reported that cross-linked Au NPs aggregated on electrode surfaces

* To whom correspondence should be addressed. Tel.: +86-532-84022750. Fax: +86-532-84022750. E-mail: shushzhang@126.com; dingcaifeng2003@163.com.

(1) Service, R. F. *Science* **1998**, *282*, 399–401.
(2) Butler, J. M. *J. Forensic Sci.* **2006**, *51*, 253–265.
(3) Staudt, L. M. *Trends Immunol.* **2001**, *22*, 35–40.
(4) He, L.; Musick, M. D.; Nicewarner, S. R.; Salinas, F. G.; Benkovic, S. J.; Natan, M. J.; Keating, C. D. *J. Am. Chem. Soc.* **2000**, *122*, 9071–9077.
(5) Bailey, R. C.; Nam, J. M.; Mirkin, C. A.; Hupp, J. T. *J. Am. Chem. Soc.* **2003**, *125*, 13541–13547.
(6) Cao, Y. W. C.; Jin, R.; Mirkin, C. A. *Science* **2002**, *297*, 1536–1540.
(7) Ginger, D. S.; Cao, Y. C.; Mirkin, C. A. *Biophotonics Int.* **2003**, *10*, 48–51.
(8) Park, S. J.; Taton, T. A.; Mirkin, C. A. *Science* **2002**, *295*, 1503–1506.
(9) Taton, T. A.; Mirkin, C. A.; Letsinger, R. L. *Science* **2000**, *289*, 1757–1760.
(10) Storhoff, J. J.; Marla, S. S.; Bao, P.; Hagenow, S.; Mehta, H.; Lucas, A.; Garimella, V.; Patno, T.; Buckingham, W.; Cork, W.; Muller, U. R. *Biosens. Bioelectron.* **2004**, *19*, 875–883.
(11) Wang, J.; Polsky, R.; Xu, D. *Langmuir* **2001**, *17*, 5739–5741.
(12) Rosi, N. L.; Mirkin, C. A. *Chem. Rev.* **2005**, *105*, 1547–1562.
(13) Gerion, D.; Chen, F. Q.; Kannan, B.; Fu, A. H.; Parak, W. J.; Chen, D. J.; Majumdar, A.; Alivisatos, A. P. *Anal. Chem.* **2003**, *75*, 4766–4772.
(14) Drummond, T. G.; Hill, M. G.; Barton, J. K. *Nat. Biotechnol.* **2003**, *21*, 1192–1199.
(15) Wang, J.; Liu, G.; Merkoci, A. *J. Am. Chem. Soc.* **2003**, *125*, 3214–3215.
(16) Hansen, J. A.; Mukhopadhyay, R.; Hansen, J. O.; Gothelf, K. V. *J. Am. Chem. Soc.* **2006**, *128*, 3860–3861.
(17) Patolsky, F.; Weizmann, Y.; Katz, E.; Willner, I. *Angew. Chem., Int. Ed.* **2003**, *42*, 2372–2376.
(18) Wang, J.; Xu, D.; Kawde, A. N.; Polsky, R. *Anal. Chem.* **2001**, *73*, 5576–5581.
(19) Cai, H.; Xu, Y.; Zhu, N.; He, P.; Fang, Y. *Analyst* **2002**, *127*, 803–808.
(20) Elghanian, R.; Storhoff, J. J.; Mucic, R. C.; Letsinger, R. L.; Mirkin, C. A. *Science* **1997**, *277*, 1078–1081.
(21) Taton, T. A.; Mirkin, C. A.; Letsinger, R. L. *Science* **2000**, *289*, 1757–1760.
(22) Storhoff, J. J.; Marla, S. S.; Bao, P.; Hagenow, S.; Mehta, H.; Lucas, A.; Garimella, V.; Patno, T.; Buckingham, W.; Cork, W.; Muller, U. R. *Biosens. Bioelectron.* **2004**, *19*, 875–883.
(23) Wang, J.; Polsky, R.; Xu, D. *Langmuir* **2001**, *17*, 5739–5741.

10.1021/ac800847r CCC: $40.75
Published on Web 08/30/2008

provided a conductive matrix for the activation of the electrochemical response of the incorporated methylene blue, and this enabled the amplified electrochemical detection of DNA.[24] Fan et al. developed a novel nanoparticle-based electrochemical DNA detection approach based on a "sandwich" detection strategy, which involved capture probe DNA immobilized on a gold electrode and signal DNA probe labeled with gold nanoparticles that flanked the target DNA sequence. This DNA sensor could detect as low as femtomolar DNA targets.[25] Au NPs conjugated to "i-motif" DNA that behaved like a pH-dependent switch that underwent reversible aggregations, which could be easily visualized by the naked eye, was reported by Sharma et al.[26] Au NPs had also been used as colorimetric indicators for aptamer-based detection of DNA-binding molecules, such as proteins, adenosine, or cocaine, by exploiting hybridization between the oligonucleotide probes immobilized on Au NPs and sequence-specific DNA strands.[27−29]

Chemiluminescence (CL) strategy is not only an important methodology for the detection of DNA hybridization based on measuring the specific luminescence activity of the labels, such as enzymes linked to the DNA probe,[30−34] but also one of the most sensitive techniques for trace analysis of metal ions with a very simple instrumentation.[35] Therefore, it should be believed that the CL technique possesses great potential for the highly sensitive detection of DNA hybridization using metal NPs as labels of an oligonucleotide probe instead of enzyme labels, which can overcome the inherent poor stability of the enzyme. Recently, Li's group[36] developed a new CL scheme for the detection of DNA hybridization based on a silver nanoparticle label with a detection limit of 5 fM. However, it is a time-consuming and complicated method. The step of Ag NPs labeled to DNA probe needs 116 h. The coupling CL reaction ($Ag-Mn-K_2S_2O_8-H_3PO_4-$luminol) employed to measure Ag^+ released from dissolution of silver NP probes was performed at 90 °C for 7 min.

Meanwhile, the chemiluminescence methods are not stable for the determination of Au^{3+} and Ag^+ because of their instability in aqueous solution, which limits the application of Au and Ag NPs as labels in the determination of DNA sequence.[37] The stability of Cu^{2+} in aqueous solution makes the CuS tags more convenient for chemiluminescence detection of a DNA sequence.

Our group reported a biosensor for the determination of short-sequence DNA based on a flow injection (FI) CL system of luminol$-H_2O_2-Cu^{2+}$ based on CuS tags.[38] However, the detection limit of 5.5×10^{-13} M was not low enough. In the present work, we developed a more sensitive nanoparticle-based DNA sensor by exploiting the signal amplification feature of Au NPs as well as the preconcentration of cupric ions dissolved from the other terminal of the oligonucleotide labeled with CuS NPs. Thus, with the sensitive measurement of cupric ions released from the hybrids by the same standard CL reaction system of luminol$-H_2O_2-Cu^{2+}$, the detection limit of target DNA (3σ) was estimated to be as low as 4.8 fM. The sensitivity was increased 6 orders of magnitude over that of the gold nanoparticle-based colorimetric method[20] and is comparable to that of the SERS method.[12,39] Moreover, the CL reaction of luminol$-H_2O_2-Cu^{2+}$ in this assay offers the obvious advantages of being simpler and faster compared with Li's work.[36] Additionally, CuS tags used in the experiment can be prepared easily at room temperature. To the best of our knowledge, it is the first time that Au NPs and CuS NPs were used as labels simultaneously, and this is the most sensitive CL method for the DNA detection with the merits of being simple and fast. Therefore, it is believed that this CL technique possesses great potential for the highly sensitive detection of DNA hybridization and immunoassay.

EXPERIMENTAL SECTION

Reagents. All of synthetic oligonucleotides were purchased from SBS Genetech. Co. Ltd. Their base sequences are as follows: capture DNA sequence, 5′-TGG AAA ATC TCT AGC AGT CGT-$(CH_2)_6$-SH-3′; target DNA sequence, 5′-ACT GCT AGA GAT TTT CCA CAC TGA CTA AAA GGG TCT GAG GGA-3′; signal DNA probe sequence, 5′-SH-$(CH_2)_6$-ATG TCC CTC AGA CCC TTT-$(CH_2)_6$-NH_2-3′; the two-base mismatched DNA sequences, 5′-ACT GCT AGA GAT TTT CCA CAC TGA CTA AAA G*C*G TCT G*T*G GGA-3′; the non-cDNA sequences, 5′-ACT GCT AGA GAT TTT CCA CAC TGA CTA CTT CAA CAG TGC CCC-3′.

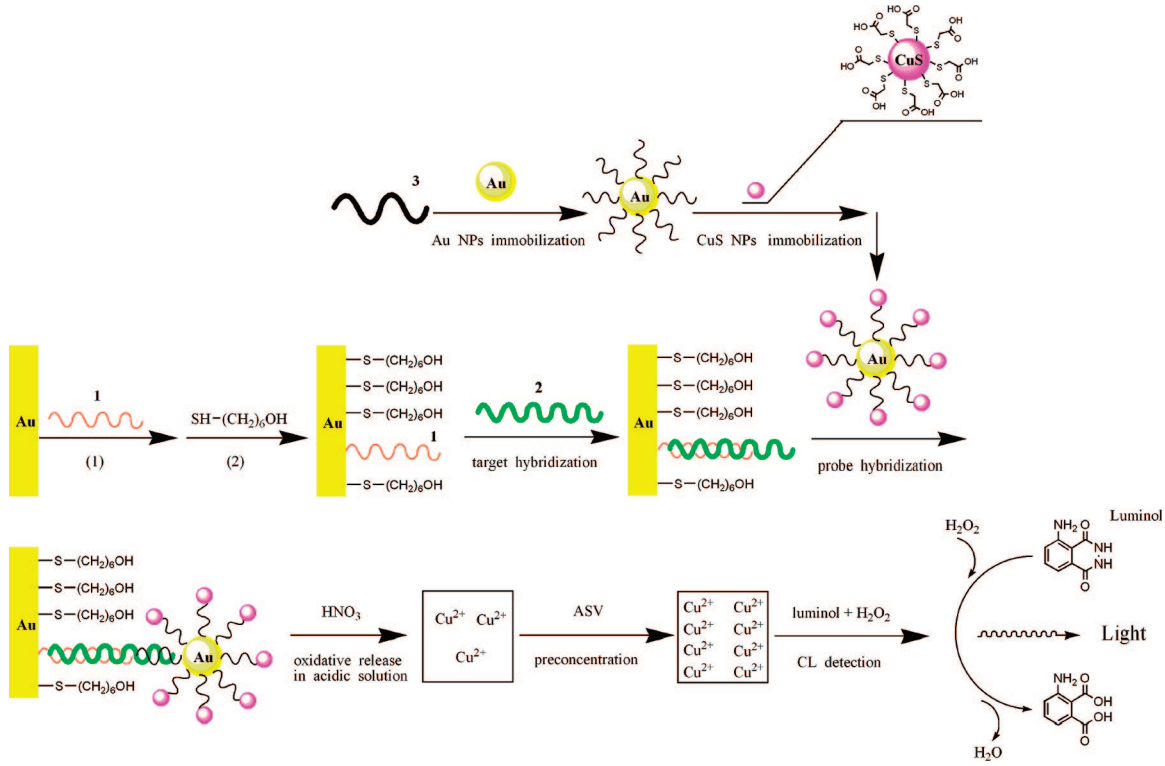
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Sigma. Luminol was purchased from ABCR GmbH & Co. Imidazole was obtained from Guoyao Chemical Co. Mercaptoacetic acid was obtained from Yuanhang Chemical Co. 6-Mercapto-1-hexanol (MCH) was obtained from Fluka. All the reagents were analytical grade and used without further purification.

A luminol stock solution of 1.5×10^{-2} M was prepared by dissolving 0.2657 g of luminol in 100 mL of 0.5 M NaOH solution; the luminol solution used in the experiment was diluted by 0.1 M NaOH$-NaHCO_3$ buffer (pH 11.0). The 0.1 M PBS buffer (pH 7.4), 0.1 M tris-HCl buffer (pH 6.8), and 0.1 M phosphate buffer containing 1‰ SDS (pH 7.0) were prepared by standard methods. Deionized and doubly distilled water was used throughout.

Apparatus. The electrochemical measurements for cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were carried out on a CHI 660C electrochemical working station (CH Instrument Co.) using a three-electrode system that consisted of a platinum wire as auxiliary electrode, an Ag/AgCl electrode as reference electrode, and a 4-mm-diameter Au disk

(24) Li, D.; Yan, Y.; Wieckowska, A.; Willner, I. *Chem. Commun.* **2007**, *34*, 3544–3546.
(25) Zhang, J.; Song, S. P.; Wang, L. H.; Wu, H. P.; Pan, D.; Fan, C. H. *J. Am. Chem. Soc.* **2006**, *128*, 8575–8580.
(26) Sharma, J.; Chhabra, R.; Yan, H.; Liu, Y. *Chem. Commun.* **2007**, *5*, 477–479.
(27) Liu, J.; Lu, Y. *Angew. Chem., Int. Ed.* **2006**, *45*, 90–94.
(28) Famulok, M.; Mayer, G. *Nature* **2006**, *439*, 666–669.
(29) Han, M. S.; Lytton-Jean, A. K. R.; Oh, B. K.; Heo, J.; Mirkin, C. A. *Angew. Chem., Int. Ed.* **2006**, *45*, 1807–1810.
(30) Fumiko, K.; Yukari, K.; Yuriko, T.; Hideji, T.; Setsuo, H.; Masafumi, Y. *Anal. Biochem.* **2005**, *341*, 77–82.
(31) Xiao, Y.; Pavlov, V.; Gill, R.; Bourenko, T.; Willner, I. *Chem. BioChem.* **2004**, *5*, 374–379.
(32) Pavlov, V.; Xiao, Y.; Gill, R.; Dishon, A.; Kotler, M.; Willner, I. *Anal. Chem.* **2004**, *76*, 2152–2156.
(33) Niazov, T.; Pavlov, V.; Xiao, Y.; Gill, R.; Willner, I. *Nano Lett.* **2004**, *4*, 1683–1687.
(34) Chen, Y.; Chen, Y. L.; Li, H.; Lu, Z. H. *Clin. Chim. Acta* **2000**, *298*, 45–53.
(35) Fan, A. P.; Lau, C.; Lu, J. Z. *Anal. Chem.* **2005**, *77*, 3238–3242.
(36) Liu, C. H.; Li, Z. P.; Du, B. A.; Duan, X. R.; Wang, Y. C. *Anal. Chem.* **2006**, *78*, 3738–3744.
(37) Imdadullah; Fujiwara, T.; Kumamaru, T. *Anal. Chem.* **1993**, *65*, 421–424.
(38) Ding, C. F.; Zhong, H.; Zhang, S. S. *Biosen. Bioelectron.* **2008**, *23*, 1314–1318.
(39) Ginger, D. S.; Cao, Y. C.; Mirkin, C. A. *Biophotonics Int.* **2003**, *10*, 48–51.

Scheme 1. Chemiluminescence Detection of DNA Hybridization through Two Steps of Amplification[a]



[a] Capture DNA sequence (1): 5′-TGG AAA ATC TCT AGC AGT CGT-$(CH_2)_6$-SH-3′, Target DNA sequence (2): 5′-ACT GCT AGA GAT TTT CCA CAC TGA CTA AAA GGG TCT GAG GGA-3′, Signal DNA probe sequence (3): 5′-SH-$(CH_2)_6$-ATG TCC CTC AGA CCC TTT-$(CH_2)_6$-NH_2-3′.

electrode as working electrode. CL emission was detected with a FI-CL instrument (IFFM-E, Remex Analytical Instrument Co. Ltd., Xian, China) that consisted of two peristaltic pumps (P1, P2), two mixture valves, and a switching valve, which was connected with an ASV cell and a CL detector according to our former work.[38] All components in the flow system were connected by poly(tetrafluoroethylene) tubing (0.8-mm i.d.). Electrochemical preconcentration performed in the ASV cell was carried out on a CHI 832B electrochemical analyzer (CH Instrument Co.) with a three-electrode system consisting of a platinum wire as an auxiliary electrode, an Ag/AgCl electrode as reference electrode, and a platinum plat electrode as working electrode with an area of 2.5 mm × 8 mm. UV−visible spectra were carried out on a Cary 50 UV−vis−NIR spectrophotometer (Varian). Transmission electron microscopy (TEM) image was taken with a JEOL JSM-6700F instrument (Hitachi).

Preparation of Au NPs. Au NPs were prepared according to the method reported previously with a slight modification.[40] $HAuCl_4$ and trisodium citrate solutions were filtered through a 0.22-μm microporous membrane filter prior to use, and then 1.0 mL of 1% trisodium citrate was added to 100 mL of boiling 0.01% $HAuCl_4$ solution and stirred for 10 min at the boiling point. The final Au NPs prepared by this method have an average diameter of ~20 nm as measured by TEM (Figure S1A in the Supporting Information). Assuming spherical nanoparticles and density equivalent to that of bulk Au (19.30 g/cm^3), an average particle mass was calculated (4.86×10^7 g/mol). The Au ion concentration in a solution of Au NPs was determined by a oxidative Au metal

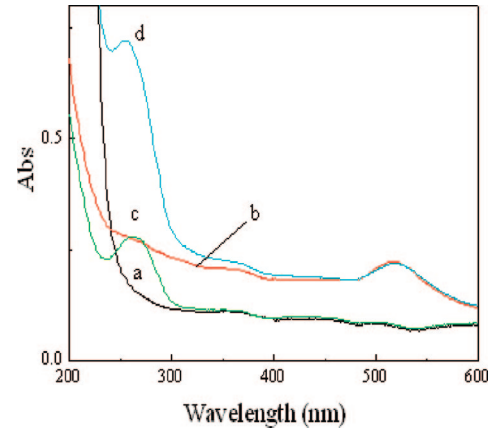


Figure 1. UV spectra of CuS colloid (a), Au colloid (b), probe DNA (c), and probe sequence difunctionalized with CuS NPs and Au NPs on the 3′- and 5′- ends, respectively (d).

dissolution-based CL method of luminol−$AuCl_4^-$ according to the reference with a slight modification.[35] Briefly, a 1 mL of DNA-modified Au NPs solution was mixed with 1 mL of 0.01 M HCl−0.1 M NaCl−0.25 mM Br_2 solution and incubated for 10 min to make sure that the Au dissolution was complete. The mixture was placed in the oven at 60 °C for 20 min to remove any remaining bromine. A 100-μL aliquot of the solution was transferred into glass tubes containing 100 μL of 1 μM luminol in 1 M NaOH, and the CL signal was displayed in the luminescence analyzer. A gold ion standard solution was used for calibration. Comparison of Au ion concentration in the Au NP solution to the average particle volume obtained by TEM analysis yielded the molar concentration of Au

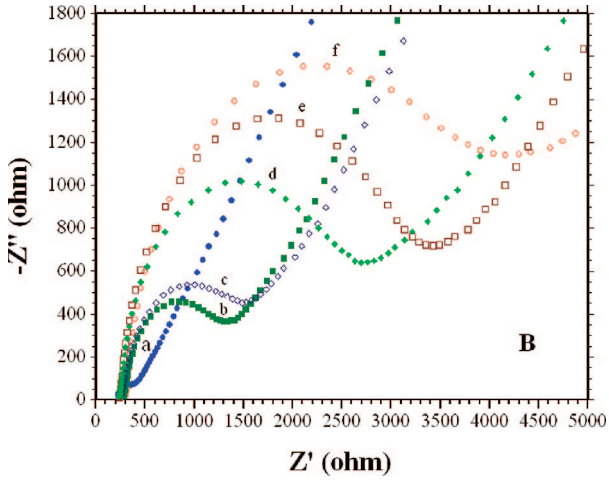


Figure 2. Electrochemical impedance spectroscopy of different electrodes. (a) The bare Au electrode; (b) the capture DNA and cysteamine-modified Au electrode; (c) after the hybridization of the target DNA with the capture DNA assembled on the surface of the Au electrode; (d−f) after the other end of target DNA was recognized by unmodified signal DNA probe (d), Au NP functionalized signal DNA probe (e), and difunctionized signal DNA probe with Au and CuS NPs (f). Supporting electrolyte, 10 mM pH 7.4 PBS containing 2.5 mM $Fe(CN)_6^{4-/3-}$ and 0.1 M KCl; scan rate, 100 mV/s.

NP in a given preparation, typically ~6.77×10^{-10} M.[41,42] The prepared colloid gold NPs were stored in brown glass bottles at 4 °C.

Preparation of Water-Soluble CuS NPs. CuS NPs were prepared according to the literature by using mercaptoacetic acid as the stabilizer.[43] Briefly, 3 μL of mercaptoacetic acid was added to 50 mL of 0.4 M $Cu(NO_3)_2$ solution, and the pH of the mixture was adjusted to 9.0 with 0.5 M NaOH solution. After being bubbled with N_2 for 30 min, 50 mL of 1.34×10^{-3} M Na_2S solution was added dropwise to the solution. The reaction was carried out for 24 h under N_2 bubbling, and a brown colloid was formed gradually. The CuS NPs have an average diameter of ~5 nm measured by TEM as shown in Figure S1B in the Supporting Information.

Assuming spherical NPs and density equivalent to that of bulk CuS (4.60 g/cm^3), an average particle mass was calculated (1.81×10^5 g/mol). The concentration of cupric ions dissolved from CuS NPs loaded on the sensors in the solution was determined by flow injection CL method as described in the paper without preconcentration. Comparison of cupric ions concentration in the solution to the average particle volume obtained by TEM analysis yielded the molar concentration of CuS NPs in a given preparation, typically ~1.39×10^{-7} M.

Modification of Signal DNA Probe with Au NPs and CuS NPs. The oligonucleotide-modified Au NPs were prepared according to the reference with a slight modification.[44] Briefly, ~3 mL of the prepared gold colloid solution was added to 2 mL of

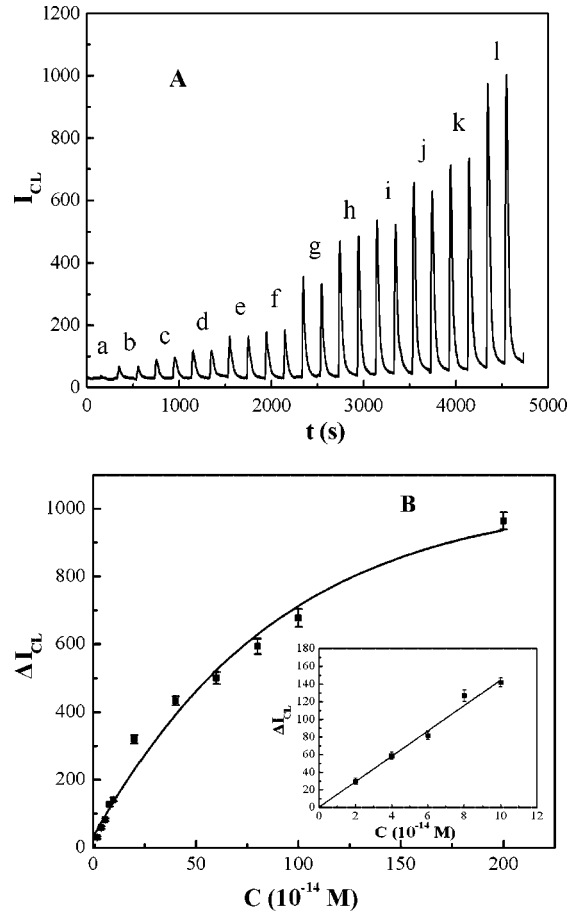


Figure 3. CL signals for duplicate measurement of cupric ions dissolved from different hybrids. The concentrations of target DNA in (A): (a) 0, (b) 2.0×10^{-14}, (c) 4.0×10^{-14}, (d) 6.0×10^{-14}, (e) 8.0×10^{-14}, (f) 1.0×10^{-13}, (g) 2.0×10^{-13}, (h) 4.0×10^{-13}, (i) 6.0×10^{-13}, (j) 8.0×10^{-13}, (k) 1.0×10^{-12}, and (l) 2.0×10^{-12} M. (B) is the calibration curves; inset is the amplification of the dots 1−5 in (B).

1.022×10^{-7} M signal DNA probe solution. Meanwhile, the same amount of signal DNA probe and 3 mL of water were mixed together, which was treated in the same way as the Au NP solution in order to determine the total amount of signal DNA probe through measuring the absorbance at 260 nm. After shaking gently for 16 h, the solution was allowed to stand for another 40 h, followed by centrifugation for at least 30 min at 10 000 rpm to remove excess reagents. Following removal of the supernatant, the red oily precipitate was washed with 5 mL of 0.1 M pH 7.0 phosphate buffer containing 0.1 M NaCl, recentrifuged, and redispersed in 5 mL of 0.1 M pH 7.0 phosphate buffer containing 0.3 M NaCl. The absorbance of the supernatant was measured at 260 nm to obtain the amount of the nonbound signal probe DNA. The number of DNA molecules immobilized on the Au NPs can be quantitatively calculated from the absorbance difference at 260 nm between the DNA solution before immobilization and the supernatant after immobilization and NP removal. The number of immobilized DNA on each NP was calculated based on the ratio of the total number of immobilized DNA to the total number of NPs in solution. The average coverage of an Au nanoparticle was calculated (~101 oligonucleotide units/nanoparticle).[36,45] The

(40) Grabar, K. C.; Smith, P. C.; Musick, M. D.; Davis, J. A.; Walter, D. G.; Jackson, M. A.; Guthrie, A. P.; Natan, M. J. *J. Am. Chem. Soc.* **1996**, *118*, 1148–1153.
(41) Lytton-Jean, A. K. R.; Mirkin, C. A. *J. Am. Chem. Soc.* **2005**, *127*, 12754–12755.
(42) Demers, L. M.; Mirkin, C. A.; Mucic, R. C.; Reynolds, R. A., III; Letsinger, R. L.; Elghanian, R.; Viswanadham, G. *Anal. Chem.* **2000**, *72*, 5535–5541.
(43) Milica, T. N.; Mirjana, I. C.; Veana, V.; Olga, I. M. *J. Phys. Chem.* **1990**, *94*, 6390–6396.
(44) Storhoff, J. J.; Elghanian, R.; Mucic, C. R.; Mirkin, A. C.; Letsinger, L. R. *J. Am. Chem. Soc.* **1998**, *120*, 1959–1964.
(45) Wang, Y. S.; Liu, B. *Anal. Chem.* **2007**, *79*, 7214–7220.

Table 1. Comparison between the Proposed CL Assay and Other Reported Techniques for the Detection of DNA Hybridization[a]

format	label	techniques[c]	detection limit of ssDNA	no. of steps
nanoparticle and nanostructure-based methods for DNA hybridization	Au nanoparticles (cross-linked)[20]	colorimetric	~10 nM	2
	Au nanoparticles (cross-linked)[24]	electrochemical	100 fM	4
	Au NPs (non-cross-linked)[48]	colorimetric	60 nM	2
	Au NPs[11]	laser diffraction	~50 fM	4
	Au NPs[10]	SPR	10 pM	4
	Au NPs[18]	PSA	15 nM	6
	Au NPs[25]	chronocoulometric	10 fM	6
	Au NPs with Ag amplification[21,22]	Scanometric	50 fM	5
	Au NPs with Ag amplification[12]	Raman spectroscopy	~20 fM	5
	Au NPs with Ag amplification[14]	electrical	500 fM	5
	Au NPs with Ag amplification[23]	PSA	32 pM	7
	silver NPs[19]	ASV	0.5 pM	4
	silver NPs[36]	CL	5 fM	7
	CuS NPs[37]	CL	550 fM	5
	ZnS, CdS, PbS NPs[15]	stripping voltammetry	270 pM	5
	ZnS and CdSe quantum dots[5]	fluorescence	2 nM	5
	liposome[9]	liposome-amplified electrochemical	50 fM	6
	magnetic particles[17]	magnetically amplified electrogenerated CL	8.3 aM	5
	Au NPs with Ag amplification[47]	biobar-code amplified scanometric	500 aM	8
	Au and CuS NPs[b]	CL	4.8 fM	7

[a] Some were adapted from ref 49. [b] This method. [c] PSA, potentiometric stripping analysis; ASV, anodic stripping voltammetry; SPR, surface plasmon resonance.

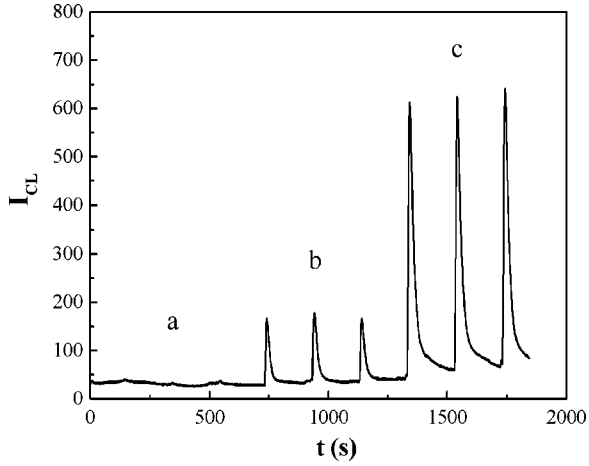


Figure 4. CL signals of luminol−H_2O_2 catalyzed by cupric ions dissolved from probe DNA hybridized with different target DNA, (a) Noncomplementary sequences; (b) two-base mismatch sequences; (c) complementary sequences. All the concentrations of target DNA in (b), (c), and (d) were 8.0×10^{-13} M.

solution of prepared Au NP functionalized oligonucleotide probes was stored at 4 °C.

A 200-μL sample of 0.1 M imidazole solution (pH 6.8) was added to 2.0 mL of 5′-amino group capped oligonucleotide-functionlized Au NP solution. After 30 min, 100 μL of 0.1 M EDC solution and 2.0 mL of CuS colloid were added to the mixture and incubated at room temperature for 24 h with gentle shaking. Finally, the signal DNA probe tagged with Au NPs and CuS NPs was collected by centrifugation at 10 000 rpm for 30 min. The precipitate was washed and then resuspended in water. The solution of signal DNA probe modified with Au and CuS NPs was stored at 4 °C for the hybridizations. The ratios of Au NPs, signal DNA probes, and CuS NPs modified on the gold electrode were ~1/101/103.

Different concentrations of DNA were used in the modification process of signal DNA probe with Au NPs and CuS NPs. From the calculation, we can see that the numbers of CuS NPs almost remain the same when the numbers of the signal DNA probe loaded on Au NPs increased slightly with the increasing of the DNA concentration from 1.022×10^{-7} to 1.022×10^{-5} M. In the present work, 1.022×10^{-7} M signal DNA probe was used throughout taking into account the economic factor.

The TEM of the signal DNA probe tagged with Au NPs and CuS NPs is shown in Figure S7 in the Supporting Information. Clearly, in the absence of signal DNA probe, only individual NPs were observed (A and B), while in the presence of signal DNA probe, aggregates of the Au NPs and CuS NPs were observed.

Fabrication of the CL Biosensor. The process for the fabrication and CL detection of the DNA biosensor is schematically shown in Scheme 1. A gold electrode was polished carefully with alumina slurries (1, 0.3, 0.05 μm) and washed ultrasonically with deionized and doubly distilled water. Then it was electrochemically cleaned in 0.5 M H_2SO_4 solution by cyclic potential scanning between 0.3 and 1.5 V until a standard CV was obtained. Subsequently, the gold electrode was rinsed with deionized and doubly distilled water and absolute ethanol in turn.

The DNA self-assembly process was performed under potential control because it was shown that the application of low positive potentials to the gold surface accelerates the chemisorption process and may assist in organizing the monolayer.[45] The clean gold electrode was initially subjected to 0.3 V for 5 min in order

(46) Ma, F.; Lennox, R. B. *Langmuir* **2000**, *16*, 6188–6190.
(47) Nam, J. M.; Stoeva, S. I.; Mirkin, C. A. *J. Am. Chem. Soc.* **2004**, *126*, 5932–5933.
(48) Sato, K.; Hosokawa, K.; Maeda, M. *J. Am. Chem. Soc.* **2003**, *125*, 8102–8103.
(49) Rosi, N. L.; Mirkin, C. A. *Chem. Rev.* **2005**, *105*, 1547–1562.

to ensure a reduced gold surface. Subsequently, the electrode was immersed in 1 mL of 0.1 M tris-HCl buffer containing 0.025 μM capture DNA and incubated for 5 min under an applied potential of 0.4 V. In order to avoid consequent nonspecific adsorption in the following hybridization steps, the modified electrode was immersed in the sodium phosphate solution containing 0.2 mM MCH for 1 h to block the uncovered gold surface. The sandwich-type format assay used consists of two steps. First, the modified electrode was immersed into 0.1 M PBS buffer containing target DNA with the different concentrations at 37 °C. One hour later, the modified electrode was taken out and immersed into 0.1 M PBS buffer containing signal DNA probe functionalized with CuS and Au NPs on the 3′- and 5′-end for 12 h. Rinsing the electrode surface with 0.1 M phosphate buffer containing 1‰ SDS (pH 7.0) after each step of the fabrication process is very important to remove nonspecifically adsorbed sequences.

FI-CL Detection. The Au electrode modified with sandwich-type hybrids labeled with Au and CuS NPs was immersed into a colorimetric tube containing 200 μL of 0.2 M nitric acid solution for 5 min. The CuS NPs anchored on the hybrids were dissolved immediately. The volume of the solution was adjusted to 1 mL with deionized and doubly distilled water. The above solution was introduced into the flow line by P2 at a constant speed of 0.6 mL/min when the direction of the switching valve was in the load position. And at the same time, deposition of copper was performed on the flat platinum electrode at −0.8 V for 100 s. During the next step of 10 s, 400 μL of nitric acid solution (pH 5.0) was drawn into the ASV cell. Then cupric ions were stripped out from the platinum plat electrode surface at the potential of +0.3 V in the third step (10 s). In the following step of 15 s, the direction of the switching valve was changed to the detection position. P1, P2, and the switching valve started to work simultaneously, the solution containing the preconcentrated cupric ions was pumped by P2, and the solution of 1.5×10^{-3} M luminol and 0.4 M H_2O_2 were pumped by P1 into the flow lines, respectively. During the last step of 65 s, the direction of the switching valve was changed to the original position, and cupric ions were reacted with the mixture of luminol and H_2O_2 in the flow cell to produce CL signal. The concentration of target DNA was quantified based upon the concentration of dissolved cupric ions, which was quantified by the CL intensity.

RESULTS AND DISCUSSION

Fabrication of the Biosensor and the Detection Process. Scheme 1 shows the method used for the amplified sensing of target DNA. A sandwich-type detection strategy involves thiol-functionalized capture DNA self-assembled on Au electrode and signal DNA probe labeled with CuS and Au NPs on the 3′- and 5′-terminus, both of which flank the DNA target sequence. Since a single Au NP could be loaded with hundreds of signal DNA probe strands, a significant amplification for the detection of target DNA was obtained. When cupric ions were dissolved from the hybrids, the secondary amplification was performed by preconcentration of cupric ions through ASV technology. The concentration of target DNA was monitored based upon the concentration of dissolved cupric ions, which was quantified by the CL intensity of luminol−H_2O_2−Cu^{2+}.

UV−Visible Spectra of the DNA−NP Conjugates. The UV−visible spectra of the CuS colloid, Au colloid, unmodified signal DNA probe, and bisfunctionalized signal DNA probe with CuS and Au NPs were recorded by the spectrophotometer as shown in Figure 1. Curve d exhibited both the characteristic absorbance of DNA (curve c) and the characteristic absorbance of Au colloid (curve b) at ∼520 nm. The results indicated that the CuS and Au NPs had been successfully labeled on the 3′- and 5′-terminus of signal DNA probe, respectively.

CL Behavior of the Luminol−H_2O_2 Catalyzed by Cupric Ions. The CL curves of luminol−H_2O_2 catalyzed by cupric ions dissolved from hybridized electrodes with the change of the signal DNA probe was shown in Figure S2 in the Supporting Information. Since no release of cupric ions from the hybrids recognized by Au NP functionalized signal DNA probe anchored on electrode surface, the CL intensities recorded were almost the same with the baseline of the solely luminol−H_2O_2 system. It was observed that the CL intensity was definitely increased when the signal DNA probe was labeled by CuS NPs only. This could be attributed to the fact that the cupric ions dissolved from the hybrid recognized by CuS NP functionalized signal DNA probe catalyzed the luminol−H_2O_2 system for luminescence intensity. With the introduction of bisfunctionized signal DNA probe by Au NPs and CuS NPs in the hybrids on the electrode surface, the CL intensity was found to be further enhanced largely. More CuS NPs could be loaded on the hybrids with the assistance of Au NPs as shown in Scheme 1 as compared with that in the absence of Au NPs, which was ascribed for this distinct enhanced CL intensity. Also, it was strongly evidenced that the cupric ions induced a catalytic role for CL.

Characterization of the Biosensor Fabrication. The fabrication process of the biosensor was characterized by EIS as shown in Figure 2. Curve a in Figure 2 showed the EIS of the bare Au electrode. An almost straight line was exhibited, which was characteristic of a mass diffusional limiting electron-transfer process. After the electrode was assembled with capture DNA and treated with MCH, the EIS of the assembled monolayer showed a large interfacial eT resistance (curve b in Figure 2). The hybridization with target DNA induced a larger resistance of EIS (curve c in Figure 2). After the further recognition of target DNA by signal DNA probe (curve d in Figure 2), the signal DNA probe functionalized with Au NPs (curve e in Figure 2), and the signal DNA probe difunctionalized with Au and CuS NPs (curve f in Figure 2), respectively, the EIS became larger and larger. As described above, dsDNA, Au, and CuS functionalized dsDNA could make it difficult for the electron transfer. These results were consistent with the fact that the electrode was fabricated as expected.

Sensitivity of the DNA Biosensor. The sensitivity of the DNA biosensor was detected as shown in Figure 3. The results showed that the CL intensities of luminol−H_2O_2−Cu^{2+} increased with the increase of concentration of the target DNA ranging from 2.0×10^{-14} to 2.0×10^{-12} M (Figure 3A). The nonlinear function for target DNA was $I_{CL} = -0.0222C^2 + 8.9747C + 46.1860$ (I_{CL} is the CL intensity; C is the concentration of target DNA, 10^{-14} M; $n = 11$, $R^2 = 0.9789$). The linear range for target DNA was 2.0×10^{-14}−1.0×10^{-13} M with the equation of $I_{CL} = 14.6290\ C + 0.1905$ (I_{CL} is the CL intensity; C is the concentration of target DNA, 10^{-14} M; $n = 5$, $R = 0.9976$; Figure 3B). A series of 11 repetitive measurements of 4.0×10^{-14} M target DNA were used for

estimating the precision, and the relative standard deviation was 3.4%. It was shown that the DNA biosensor had good reproducibility. The detection limit of 4.8×10^{-15} M target DNA could be estimated using 3σ. This method and some other amplified techniques, which can greatly improve the sensitivity of the DNA assay, are listed in Table 1. The sensitivity of this present work was found to be increased ~6 orders of magnitude over that of the gold nanoparticle-based colorimetric method and was comparable to that of Li's work using CL detection of metal nanoparticle-based DNA hybridization, which was time-consuming and complicated.

Selectivity of the DNA Biosensor. The selectivity of the present biosensor was investigated by using the signal DNA probe labeled with Au and CuS NPs to hybridize with the same concentration of complete complementary target DNA sequences, the two-base mismatched DNA sequences and the noncDNA sequences, respectively, as shown in Figure 4. A well-defined CL signal of luminol$-H_2O_2-Cu^{2+}$ was obtained for the complementary sequences. The CL intensity for two-base mismatched sequences was significantly weaker than that of the complementary sequences, and the noncomplementary sequences showed no response.

CONCLUSIONS

A novel and sensitive biosensor for the sequence-specific DNA detection based on FI-CL and signal amplification by Au NPs was reported in the present work. The concentration of target DNA was quantified according to the determination of cupric ions concentration by CL intensity of luminol$-H_2O_2-Cu^{2+}$, in which the cupric ions was released from the constructed "sandwich-type" DNA hybrids on the electrode surface. The sensitivity of the sensor was further improved by the preconcentration of the dissolved cupric ions using an ASV process. The detection limit of 4.8×10^{-15} M target DNA was obtained. The resulting CL biosensor exhibited ultrasensitivity, high selectivity, and fair stability and might be a good alternative for other bioassays. We now anticipate finding more suitable nanoparticle tags and CL reactions to fabricate more sensitive and simple DNA or immunoassay biosensors.

ACKNOWLEDGMENT

The work was supported by the Natural Science Foundation of Shandong Province (Y2006B07), the project of Science and Technology of Qingdao (06-3-1-Y-X), and the National Nature Science Foundation of China (20775038). We highly appreciate the kind English language help for our manuscript from Dr. Shufeng Liu.

SUPPORTING INFORMATION AVAILABLE

Figures for instrument for the FI-CL detection and ASV cell for electrochemical preconcentration, and the CL behavior of the luminol-H_2O_2 catalyzed by cupric ions. Rinse of the electrode surface; calculation of the ratios of Au NPs, signal DNA probes, and CuS NPs; the CL mechanism of luminol$-H_2O_2-Cu^{2+}$ system; the stability of the DNA biosensor; and the importance of the preconcentration step. The material is available free of charge via the Internet at http://pubs.acs.org.

Received for review December 29, 2007. Accepted August 4, 2008.

AC800847R