



Graphene quantum dots-based electrochemiluminescence detection of DNA using multiple cycling amplification strategy

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ABSTRACT

In this study, a novel strategy for amplified electrochemiluminescence (ECL) detection of DNA was proposed based on excellent ECL activity of graphene quantum dots (GQDs) coupled with multiple cycling amplification technique. A new type of graphene QDs with well ECL property and uniform size were firstly synthesized, then the graphene QDs were assembled on the electrode by poly (diallyldimethylammonium chloride) (PDDA)-graphene oxide (GO) nanocomposites, which could greatly improve ECL signal and stability of QDs. A novel signal-on ECL biosensor for DNA analysis was designed by using ECL quenching of gold nanoparticles (NPs) to graphene QDs combined with endonuclease-aided cyclic amplification strategy. As a result, the proposed strategy can be conveniently expanded to other biosensing application, especially in clinical diagnoses.

1. Introduction

Electrochemiluminescence (ECL) analysis has received considerable attention in analytical chemistry due to its simplicity, high sensitivity and low background [1,2]. In recent years, the ECL property of quantum dots (QDs) has attracted more and more researching interest. Compared with the conventional ECL reagents, such as luminol, ruthenium, QDs possesses many advantages, such as neutral detection conditions and easy realizability in both cathodic and anodic processes with diverse co-reactants, leading to multifarious ECL biosensing strategies [3–5]. More and more researchers are beginning to realize the potential applications of quantum dots in ECL analysis [6,7]. It is reported that QDs-based ECL technology has been widely applied to biosensors [8–11], such as DNA or cell analysis [12–14]. However, heavy metals as the essential elements in available high ECL performance semiconductor QDs exhibited the inherent toxicity, which have raised serious health and environmental concerns [15]. Thus, it is of great value to develop novel low-toxicity or nontoxic ECL nanomaterials. In recent years, graphene quantum dots (GQDs) have gradually become the focus of attention, scientists in various fields have studied them extensively. GQDs exhibited low toxicity, unique electro-optical properties, robust chemical inertness, and attractive biocompatibility [16–19].

A variety of methods have been carried out on the preparation of

graphene QDs, such as chemical solution, ultrasonic and microwave methods [20,21]. The as prepared graphene QDs have water solubility and good performance in ECL biosensors [22–24]. A novel signal-off ECL DNA biosensor based on graphene QDs was developed using hepatitis C virus-1b genotype complementary DNA (HCV-1b cDNA) as a model [24]. However, the pure QDs usually have low ECL signal and poor stability. Thus, the PDDA-functionalized graphene oxide (GO) was used to immobilize more QDs, which greatly improved ECL signal and stability of QDs. In addition, electrochemiluminescence resonance energy transfer (ECL-RET) has attracted growing attention in the detection of proteins, DNA and small molecules [25–27]. The quenching effect of gold nanoparticles to QDs ECL has good application prospect in biological research [28,29].

Nucleic acids are closely associated with some diseases, clinical diagnostics, drug screening, food safety, and environmental monitoring [30,31]. The p53 gene is one of the important tumor suppressor genes found so far, which could inhibit the growth of tumor cell and induce the apoptosis of the tumor cell [32]. Mutant mitochondrial DNA with large-scale deletions has been frequently observed in patients with mitochondrial encephalomyopathies [33]. Human papillomavirus (HPV) type 16 DNA detection revealed that infection with oncogenic genital human papillomaviruses (HPV) has been established as the main etiologic agent for cervical neoplasia [34]. Thus, it is urgently needed to develop an efficient and highly sensitive method for detection of

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disease related nucleic acids. In order to improve the detection sensitivity and realize the trace level detection, a variety of DNA related processes have been designed to amplify the signals because of the specific molecular recognition ability and good biochemical properties of DNA [35–37], such as strand displacement amplification (SDA) [38], polymerase chain reaction (PCR) [39], and endonuclease-aided amplification [40]. Recently, DNA cycling amplification technology has drawn great attention of researchers [41], and has been widely applied to electrochemistry and optical biosensors [42].

In this work, a new kind of graphene QDs with uniform size and good electrochemiluminescence were prepared, and was used to design a novel and sensitive ECL biosensor for target DNA detection by cycling amplification strategy. A large number of graphene QDs were immobilized on the electrode by using PDDA-GO complex, which could greatly improve ECL signal and stability of the electrode. Effective ECL quenching effect of AuNPs to graphene QDs was studied, which was combined with endonuclease-assisted amplification technique to develop a sensitive ECL assay method for DNA detection. The proposed ECL biosensing system displayed good selectivity and high sensitivity, and has promising application prospect in the detection of real samples.

2. Experimental section

2.1. Reagents

Trisodium citrate and chloroauric acid (HAuCl_4) were obtained from Alfa Aesar (Beijing, China). 1-ethyl-3-(3-(dimethylamino) propyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were obtained from Aladdin Reagents Co., Ltd. (Shanghai, China). Tris (hydroxymethyl) aminomethane (Tris) and EDTA were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Phi29 polymerase, endonuclease (Nt. BbvCI, Nb. BbvCI) and dNTPs were purchased from New Zealand Biotechnology (Beijing) Co., Ltd. All reagents were of analytical grade and used as received. Ultrapure water was used throughout the experiment. All the DNA used in this experiment were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). The DNA solution was prepared from TE buffer solution (pH 7.4) containing 10 mM tris-HCl (pH 8.0), 1 mM EDTA and 12.5 mM MgCl_2 . The sequences of oligonucleotides are depicted in Table 1.

2.2. Apparatus

ECL signals were measured on a MPI-A Electrochemiluminescence Analyzer (Xi'an Reomai Electronic Technology Co., Ltd.) using a gold working electrode, a saturated calomel reference electrode, and a Pt wire counter electrode. The photomultiplier tube voltage was 500–800 V. Transmission electron microscopy (TEM, JEM-2000EX) and scanning electron microscopy (SEM, JSM-6700 F) were used to characterize the graphene QDs.

2.3. Preparation of gold nanoparticles

0.1153 g of sodium citrate was dissolved in 5.0 mL of ultrapure water. 100.0 mL of ultrapure water was added to the flask and heated to boiling, then 2.0 mL of 1% HAuCl_4 solution was added to the water and kept boiling for 8 s. Then, 4.0 mL of sodium citrate solution was added

to the reaction solution, and the boiling lasted for 1 h. After the heating source was turned off, the solution was cooled down to room temperature.

2.4. Preparation of graphene QDs

2 g of citric acid was added to a 10 mL small beaker, and heated to 200 °C for 30 min until the white solid citric acid turned orange. Then, 10 mg mL^{-1} of sodium hydroxide solution was added to the beaker dropwise under vigorous stirring until the pH was 7. The resulting graphene QDs solution was obtained, and stored at 4 °C.

2.5. Preparation of AuNPs/PDDA-GO nanocomposites

0.1 mL of 10% PDDA solution was mixed with 17.0 mL of 0.62 M KCl solution, then the mixed solution was added to 4.5 mL of polyvinylpyrrolidone-coated graphene oxide solution and sonicated for 2 h. After centrifugation, the resulting solution was washed and redispersed in 5 mL of ultrapure water to obtain the PDDA-GO complex.

6.0 mL of AuNPs solution was added to the prepared PDDA-GO solution, and sonicated for 30 min. After centrifugation, the solution was washed three times and dispersed in 3.0 mL of ultrapure water to obtain the purple AuNPs/PDDA-GO solution.

2.6. ECL detection of DNA using DNA amplification technology

The gold electrode is polished with $\alpha\text{-Al}_2\text{O}_3$ of 1.0 μm , 0.3 μm and 0.05 μm in sequence, then is ultrasonically washed with ultrapure water, and dried in air. After 8 μL of AuNPs/PDDA-GO was dropped on the electrode surface and dried naturally, 7 μL of graphene QDs were added to the modified electrode, and dried naturally.

The AuNPs-DNA quenching probe was prepared as follows. 1.0 mL of AuNPs solution was centrifuged at 10,000 rpm for 10 min. After removing the supernatant, the AuNPs was redissolved in 100 μL of deionized water. 50 μL of thiol-modified hairpin DNA (1.0×10^{-5} M) was added to 100 μL of the purified AuNPs solution, then the reaction solution is shaken at 25 °C for 24 h. The resulting solution is centrifuged at 15,000 rpm for 30 min to remove unreacted DNA. After being washed with PBS, the solution is stored at 4 °C for use.

50 μL of reaction system is as follows: 10 μL of template DNA (1.0×10^{-5} M), 10 μL of target DNA (or 5% serum spiked with various-concentration target DNA), 2 μL of dNTPs (10 mM), 16 U of Bst-DNA polymerase and 12 U of endonuclease were mixed, and shaken at 37 °C for 2 h.

10 μL of AuNPs-DNA solution was dropped on the surface of the graphene QDs modified electrode, and reacted at 37 °C for 12 h. After being rinsed with deionized water, the quenched ECL signal was detected. After being slightly rinsed with double-distilled water, the electrode was immersed in the above DNA reaction system, and the endonuclease (Nb. BbvCI) was added to the reaction system. After the reaction was shaken at 37 °C for 2 h, the recovered ECL signals related to DNA concentration were measured.

3. Results and discussion

3.1. Characterization of graphene quantum dots

Fig. 1 (A) is a transmission electron microscopy (TEM) image of graphene QDs. As can be seen from the figure, the average diameter of the quantum dots is about 25–30 nm (B), the size and morphology are uniform.

3.2. ECL behavior of GQDs

Fig. 2 showed the ECL-potential curve and cyclic voltammogram (inset) of GQDs on the electrode, a strong ECL peak at -1.8 V was

Table 1
Sequences of DNA in the experiments.

Oligonucleotides	sequence (5'-to-3')
1	CACCTCAGCACTAAGGCC
2	CAGTGCATTGAGCGCTGAGGCCCTTAGTGCTGAGGTG
3	GCTGAATGCACTG
4	NH ₂ - TTT TTT TTT TTT TTT CCG GGT TGT TTA GTGC TGAGG TGC GG ACC CGG-SH

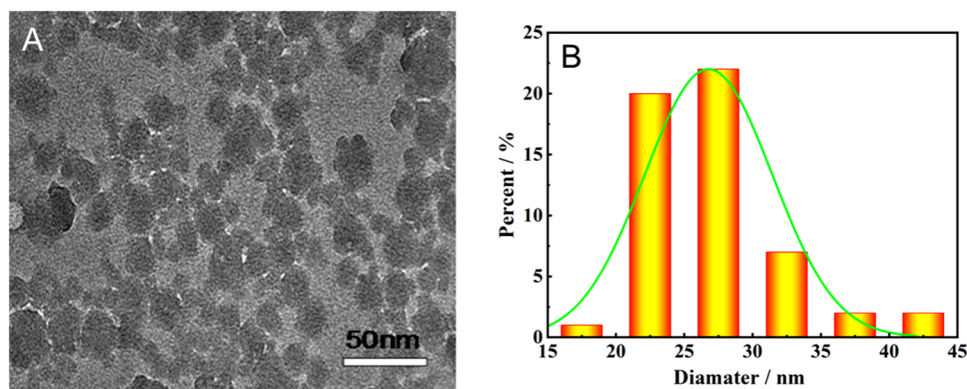


Fig. 1. (A) TEM image of GQDs, (B) Size distribution of GQDs.

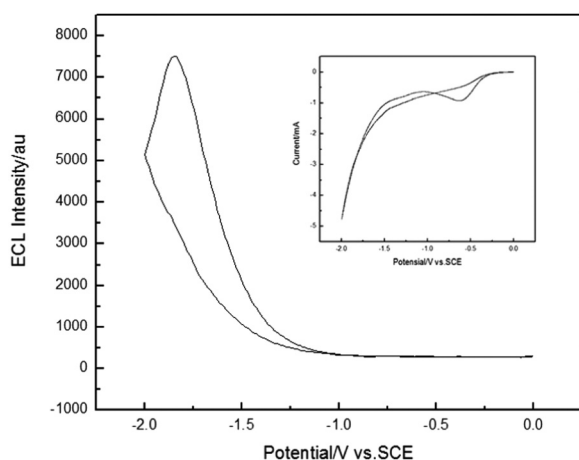


Fig. 2. ECL-potential curve and cyclic voltammogram (inset) of the GQDs on the electrode.

observed, resulting from the reaction of QDs with $S_2O_8^{2-}$. The possible ECL mechanisms are as follows [43]:

- (1) $QD + e^- \rightarrow QD^{\cdot -}$
- (2) $S_2O_8^{2-} + e^- \rightarrow SO_4^{\cdot -} + SO_4^{2-}$
- (3) $QD^{\cdot -} + SO_4^{\cdot -} \rightarrow QD^* + SO_4^{2-}$
- (4) $QD^* \rightarrow QD + h\nu$

3.3. Principle for ECL detection of DNA based on GQDs coupled with cycling amplification technique

ECL resonance energy transfer (ECL-RET) is caused by the energy transfer between ECL donor and acceptor at the nanometer-scale [44], which has attracted growing attention in the detection of proteins, DNA and small molecules [45]. In addition, ECL activity of GQDs combined with exonuclease-induced target recycling was used for sensitive ECL detection of RNA [46]. In this work, Fig. 3 showed the design principle for sensitive detection of DNA based on GQDs ECL coupled with cycling amplification technique. When the target DNA is present, it is complementary with the original template DNA. With addition of DNA polymerase, target DNA as the primer extended, releasing red DNA s1. At the same time, the formed complementary strands were specifically recognized by endonuclease and sheared by it, which further led to the release of DNA s1. With the cycling polymerization and cutting, a large number of DNA sequence s1 were released.

Meanwhile, the graphene QDs were immobilized on the electrode by PDDA-GO complex, then the Au NPs-DNA as quencher probe was linked to the electrode with QDs. ECL energy transfer between QDs and Au NPs occurred, QDs ECL were quenched. In the presence of DNA s1,

s1 hybridized with the DNA quencher probe to form a specific double-stranded DNA for endonuclease, which was then cleaved by endonuclease to release the quencher, leading to amplified ECL signal change for quantitative detection of target DNA.

3.4. Characterization of the fabrication process for ECL biosensor

Fig. 4A showed TEM image of the AuNPs-DNA, it is observed that the prepared AuNPs-DNA probe has good morphology and uniform size. It can be seen from Fig. 4B the average diameter of the AuNPs-DNA is about 11–13 nm.

As can be seen from Fig. 5, GO displayed the sheet-like structure and fewer layers. The thickness of GO was thin, and the surface is relatively smooth, suggesting that the prepared GO was successful for application in biosensor.

Fig. 6 showed the SEM images of PDDA-GO/GQDs/DNA-gold NPs on the electrode. When the DNA-gold NPs probe was assembled on the surface of PDDA-GO on the electrode, a thin film with many nanoparticles was covered on the electrode surface, indicating that both the PDDA-GO and DNA-gold NPs probe were successfully assembled on the electrode surface, and the biosensor based on ECL quenching of gold NPs to GQDs is successfully constructed.

3.5. ECL detection of DNA based on GQDs by cycling amplification technique

As is shown in Fig. 7A, almost no ECL was seen on the bare electrode (curve a), while the PDDA-GO/GQDs modified electrode displayed high ECL signal (curve b). After DNA-gold NPs quenching probe was linked to the electrode, ECL signal decreased (curve c), suggesting that gold NPs could quench QDs ECL. When target DNA was present combined with cycling amplification, the ECL signal increased (curve d), demonstrating the present strategy could be applied to target DNA detection.

Fig. 7B showed ECL signals for detecting target DNA of different concentrations. With increasing concentrations of target DNA, more DNA s1 were generated and then hybridized with the DNA-Au NPs quencher probe, which led to cleaving reaction of more quencher probe. So more Au NPs were released from the electrode, resulting in increase of ECL signal (curve b to h). The results indicate that target DNA concentration could be detected by the change of ECL signal of the method.

Fig. 8 showed the relationship between the change of ECL signal (ΔI_{ECL}) and the detected DNA concentration. It can be seen that the change of ECL signal (ΔI) gradually increased with increasing DNA concentration, ΔI is linearly proportional to the logarithm of DNA concentration in the range of 1.0 pM to 1000 nM, the detection limit is 0.1 pM. In addition, five times measurements for target DNA (0.1 nM) was repeated, the relative standard deviation (RSD) is 7.5%, indicating

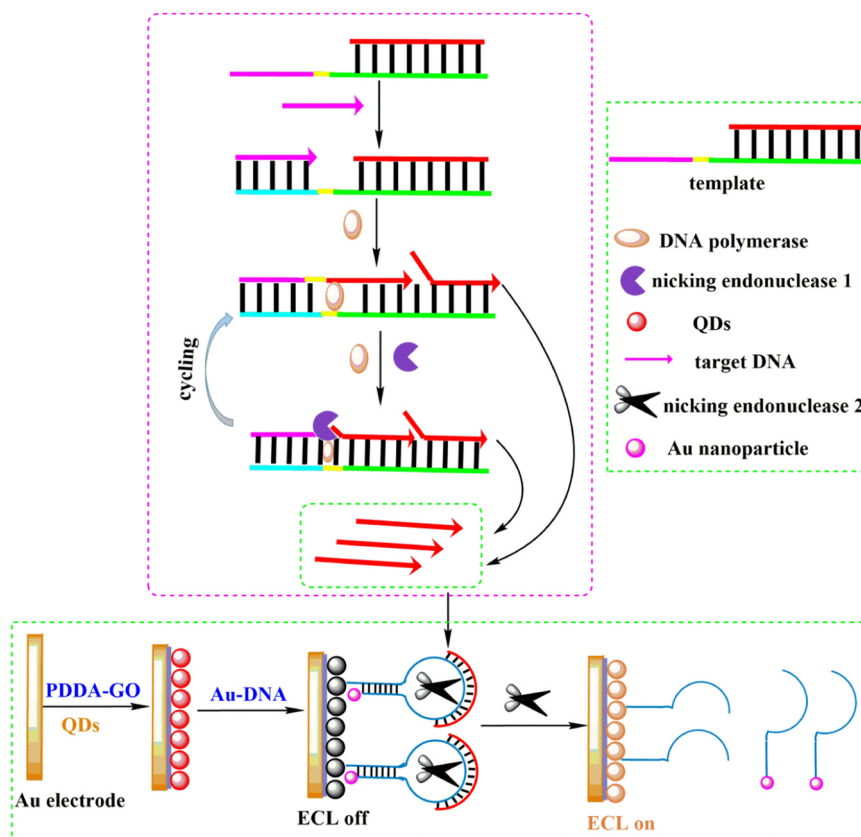


Fig. 3. Schematic representation for principle of sensitive DNA detection based on GQDs ECL coupled with cycling amplification technique.

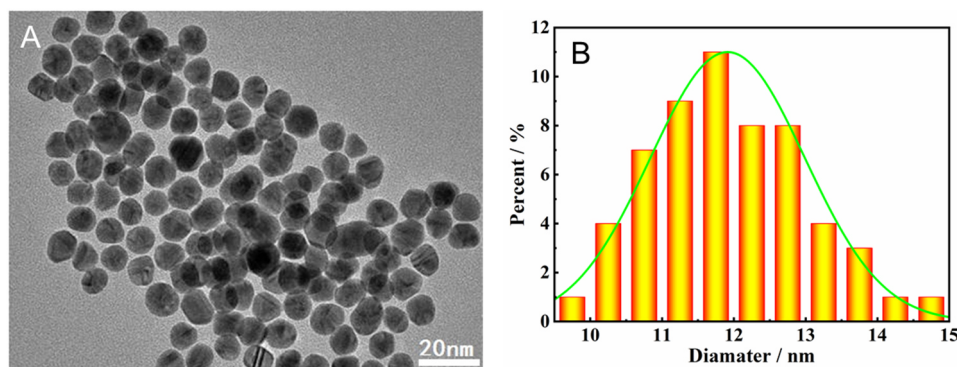


Fig. 4. (A) TEM image of the AuNPs-DNA; (B) Size distribution of AuNPs-DNA.

that the proposed method has good precision for detecting target DNA. According to the linear equation, quantitative analysis of the target DNA concentration can be achieved.

The selectivity of this method for detecting target DNA was examined. The target DNA, single base-mismatched DNA, and completely non-complementary DNA sequences at the same concentration were detected in the same method. As is shown in Fig. 9, the change of ECL response signal for target DNA (a) was obviously very high. By comparison, there is almost no obvious change for the ECL response signals to the mismatched DNA. The results demonstrated that the proposed method has good selectivity for detection of target DNA.

3.6. Application of the strategy for ECL detection of DNA based on GQDs by cycling amplification technique

To investigate the capability of the proposed method to detect the target DNA in complex biological samples, we spiked various

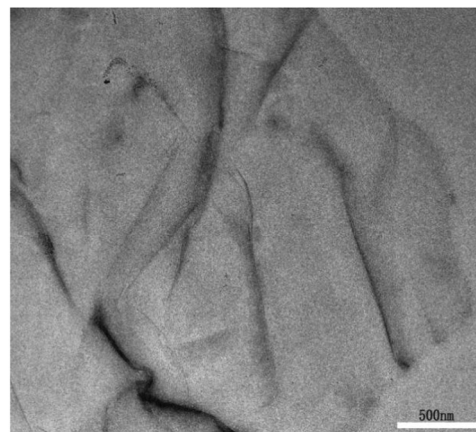


Fig. 5. Transmission electron microscopy image of GO.

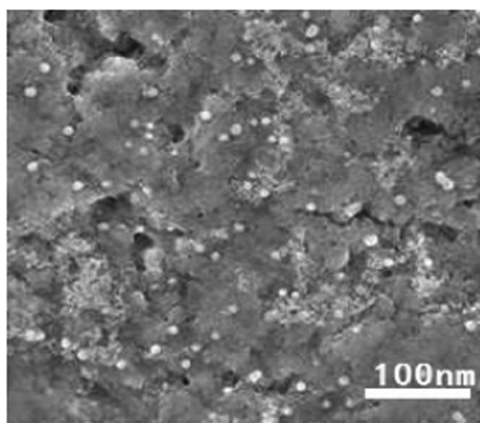


Fig. 6. SEM images of PDDA-GO/GQDs/DNA-gold NPs on the electrode.

concentrations of DNA into 5% serum samples and calculated their recovery ratios. (The corresponding results were shown in Table 2). The recoveries varied from 95.0% to 105.5%, which is acceptable. The results demonstrate that the proposed sensing method can be potentially applied to real samples.

3.7. Comparison of the ECL strategy with other detection methods

By integration of electrochemistry and spectroscopy, the proposed ECL approach has shown potential superiorities over other optical methods, such as fluorescence (FL), chemiluminescence (CL), and colorimetry. First, compared with fluorescence, ECL has no need for external light sources, which disables background responses from luminous impurities and scattered light, resulting in high sensitivity. Second, the position of ECL emission can be controlled as ECL emission is close to the surface of the electrode. Third, it has an excellent specificity to ECL species and co-reactants, and can selectively alternate the applied potential. Finally, ECL allows better control over the time of ECL systems, which could improve their simplicity and reproducibility. Therefore, the ECL sensor has become one of the most powerful analytical tools in the field of trace target detection. Table 3 showed the advantages of the present ECL sensor.

4. Conclusion

In conclusion, we prepared a novel graphene QDs with good electrochemiluminescence, and used it to design a sensitive ECL biosensor for target DNA detection by using cycling amplification technique. A new PDDA-GO complex was used to immobilize graphene QDs on the

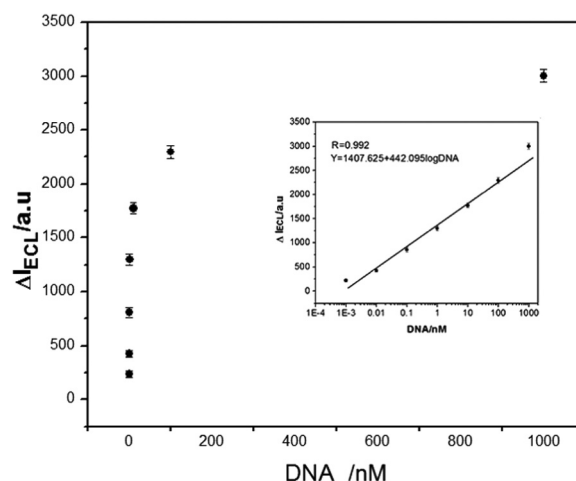


Fig. 8. Relationship between ΔI and DNA concentration. Inset : The logarithmic calibration curve for DNA detection. (1.0 pM ~ 1000 nM).

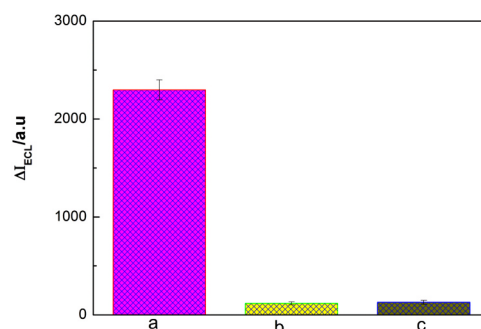


Fig. 9. ECL responses for detecting different DNA sequences (1.0×10^{-7} mol·L⁻¹). (a) Target DNA, (b) single-base mismatched DNA, (c) completely mismatched DNA. The blank was deducted.

Table 2

Recovery of DNA Assay at Different Concentrations Spiked into Serum Samples.

sample	spiked	Found	Recovery	RSD
	(pM)	(pM)	(%)	(%)
1	5.0	4.85	97.0	2.2
2	50.0	47.5	95.0	3.6
3	100.0	104.6	104.6	3.2

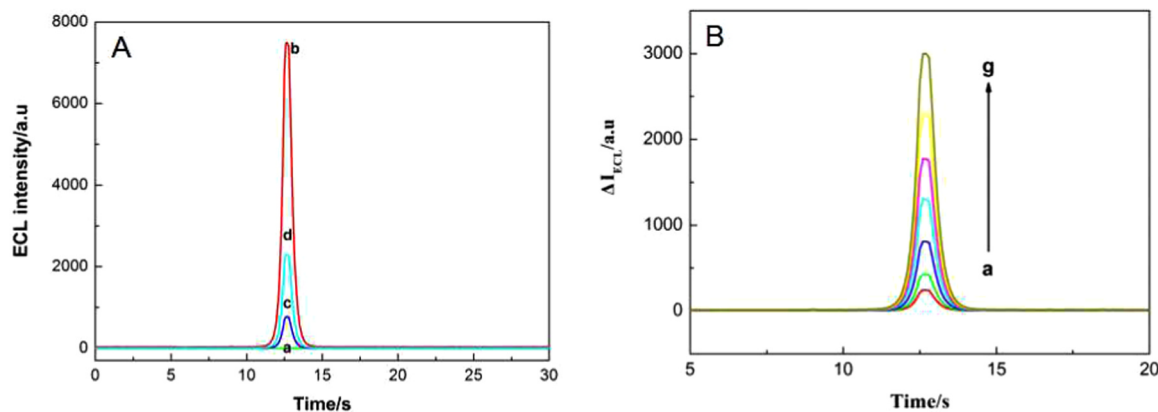


Fig. 7. (A) Feasibility of the ECL biosensor. (a) bare electrode, (b) PDDA-GO/GQDs/electrode, (c) PDDA-GO/GQDs/DNA-gold NPs/electrode, (d) PDDA-GO/GQDs/DNA-gold NPs/target DNA electrode. (B) ECL response signals for different concentrations of DNA (M). (a) 1.0×10^{-12} , (b) 1.0×10^{-11} , (c) 1.0×10^{-10} , (d) 1.0×10^{-9} , (e) 1.0×10^{-8} , (f) 1.0×10^{-7} , (g) 1.0×10^{-6} .

Table 3
Advantages of the ECL sensor over other optical methods.

Advantages	ECL	FL	CL	colorimetry
external light sources	without	with	without	without
control toward the position of the luminous emission by electrode	with	without	without	without
specificity between reactants and co-reactants	very high	low	low	very low
selectivity by applied potential	with	without	without	without
sensitivity	very high	low	high	very low

electrode, which greatly improved ECL signal and stability of the electrode. ECL quenching of AuNPs to graphene QDs was combined with cycling amplification technique to develop a sensitive ECL method for DNA assay. The ECL biosensor showed good selectivity and high sensitivity for DNA detection, which could be applied to the detection of real samples.

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