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Letters to *Analytical Chemistry*

Electrochemiluminescence Biobarcode Method Based on Cysteamine−Gold Nanoparticle Conjugates

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The recently developed DNA−gold nanoparticle (DNA−GNP) biobarcode assay provides polymerase chain reaction (PCR)-like sensitivity for nucleic acid and protein targets without a need for enzymatic amplification. However, application of the conventional assay is challenged by its complex, expensive, time-consuming, and labor-intense procedure. Herein, we present a new electrochemiluminescence (ECL) biobarcode method based on cysteamine−GNP conjugates. In this strategy, an ECL nanoprobe is fabricated that relies on GNP that is modified with tris-(2,2′-bipyridyl) ruthenium (TBR) labeled cysteamine to boost ECL signals and single strand DNA for target recognition. Specifically, a sandwich complex that consists of a biotin labeled capture probe, target DNA, and cysteamine−GNP conjugate is captured by magnetic microparticles (MMPs) and subsequently identified by the ECL signals from loaded TBR. With the use of the developed probe, a limit of detection as low as 100 fM can be achieved and the assay exhibits excellent selectivity for single-mismatched DNA detection even in human serum. The proposed ECL based method should have wide applications in diagnosis of genetic diseases due to its high sensitivity, simplicity, and low cost.

It is essential to develop precise and convenient detection methodologies that detect DNA and protein samples at extremely low concentration. This ability is critical in diagnosis of genetic, environmental monitoring, and food analysis.[1−3] During the past decade, the amplification of the DNA−gold nanoparticle (GNP) biobarcode assay has shown promise in the development of powerful tools for nucleic acid and protein detection.[4−10] The typical biobarcode assay relies on two types of particles. One is GNP functionalized with a large number of oligonucleotide strands (the barcodes) and a corresponding recognition agent. The other is magnetic microparticle (MMP) encoded with single-component oligonucleotides that specially bind to the targets. The barcode strands released from the GNP surfaces can be used as a means of amplification to quantitatively detect the target. The assay has exhibited polymerase chain reaction (PCR)-like sensitivity for both protein and nucleic acid without a need for enzymatic amplification. However, some draw backs of the conventional biobarcode assay are its high assay costs, sophisticated instruments, and lengthy experimental procedures, which limit its practical application. To overcome these drawbacks, many developed biobarcode methods have been reported recently in conjugation with electrochemical,[11−14] colorimetric,[15] fluorimetric,[16] and chemiluminescent[17] techniques.

Until now, electrochemiluminescence (ECL) that represents a marriage between electrochemical and chemiluminescent methods has become an important and powerful tool in analytical and clinical application owing to its simplicity, low cost, and high

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sensitivity.[18−20] Moreover, the combination of MMPs with tris-(2,2′-bipyridyl) ruthenium (TBR) and tripropylamine (TPA) reaction has been demonstrated to considerably increase ECL sensitivity and simplify the detection process in our previous studies.[21−23] Recently, our group developed a new class of PCR-free methods based upon the combination of ECL and the DNA−GNP biobarcode method for genetically modified organism (GMO) and telomerase activity.[24,25] The ECL biobarcode assays based on DNA−GNP conjugates eliminate the release of barcode DNA probes from the target−nanoparticle complex and the repeated hybridization of the probes for quantification, thus simplifying the analytical procedure. However, the current design, which typically employs TBR-labeled oligonucleotide to amplify ECL signals, has several disadvantages. First, a double labeling oligonucleotide is required, which is synthetically demanding and costly. Second, to maximize oligonucleotides loading on the GNPs, it is necessary to reach the final concentration of NaCl as high as 1.0 M by adding gradually and then an incubation of 1 or 2 days is required. Finally, the resulting GNP probe is susceptible to salt concentrations, surfactants, and nucleases, thus increasing variability and restricting the scope of its application.

It has been reported that thiols are among the most successful chemicals employed for an attachment to metals via forming a strong metal−sulfur chemical bond.[26,27] In the present work, we describe the development of a new version of the ECL biobarcode assay, in which cysteamine instead of double labeling oligonucleotides is used as a new kind of biobarcode. It is well-known that cysteamine, a kind of sulfur containing biomolecule, is frequently used both as the stabilizer for GNPs[28] and as a linking agent at GNP surfaces.[29,30] In this system, the sulfur atoms of the cysteamine are applied to bind to the gold surface while the amino groups are employed for the attachment of TBR. We have demonstrated the utilizing of cost-effective cysteamine could overcome disadvantages of conventional ECL signal probe, simplifying analytical procedure and shortening analysis time. This novel ECL assay based on cysteamine−GNP biobarcode amplification can quantitatively detect DNA with high speed and sensitivity, and the detection limit of the current work is as low as 100 fM. Furthermore, it demonstrates excellent specificity for single-base mismatched and perfectly matched oligonucleotides even in human serum. We expect the combination of cysteamine, GNP, and MMP based ECL technology opens new perspectives in the development of tools for analytical chemistry.

For the proof-of-concept experiment reported herein, a segment of p53 gene (5′-GAGGTGCGTG TTTGTGCCTG TCCTGGGAGA GA-3′) was chosen as our initial target for its important role in cell-cycle arrest and DNA repair mechanisms.[31] A custom-built ECL detection system was described in detail in our previous research.[21] In our study, the approach was also based on two types of nanoparticles (Figure 1). The first one is streptavidin-coated MMP that could function with capture probe 1 (5′-biotin-TTT TTT TTT TTC TCT CCC AGG ACA G-3′). Capture probe 1 labeled with biotin is complementary to one end of the target DNA. The second one, the ECL nanoprobe, is comprised of GNP that are modified with two types of molecules, one is the capture probe 2 (5′-ACA AAC ACG CAC CTC TTT TTT TTT TTT-$(CH_2)_3$-SH-3′) for target recognition and the other is TBR labeled cysteamine to enhance ECL signals (Figure 1A). The thiol modified capture probe 2 is complementary to the other end of the target DNA. The TBR-*N*-hydroxysuccinimide (NHS) ester was synthesized by our lab (see the Supporting Information).[32]

Two facile steps of constructing ECL nanoprobe are illustrated in Figure 1A (details in the Supporting Information). Briefly, the first step was to load cysteamine and thiolated capture probe 2 on GNPs and then mix thiols modified GNPs with TBR-NHS esters and incubate the mixture for 10 h. The resulting ECL nanoprobes are separated by centrifugation. The success of the biobarcode amplification is closely related to the loading quantity of the cysteamine conjugated with TBR. Therefore, to construct the optimal ECL nanoprobes, the effect of the different cysteamine-to-capture probe 2 ratios from 100:1, 10:1, and down to 1:1 on ECL intensities of ECL nanoprobes was investigated. Namely, 1 mL of 11 nM citrate-stabilized GNPs were incubated with a constant concentration (0.72 μM) of capture probe 2 and 0.72, 7.2, and 72 μM cysteamine, respectively. Figure 2 shows, when the cysteamine-to-capture probe 2 ratio is 10:1 or 100:1, it would induce GNPs to self-assembly accompanied by a large red shift in the plasmonic adsorption (Figure 2A) and sequentially influence the combination of the NH_2 group and TBR, reducing the ECL intensity of ECL nanoprobes (Figure 2B). The reason of it may be that the NH_2 groups interacted with solution ions have a tendency to be adsorbed on the surface of GNPs,[33,34] and the high cysteamine concentration could urge self-assembly of the GNPs.[35] However, the lower ratio of cysteamine-to-capture probe 2 would decrease the cysteamine loading and diminish ECL signals. When these issues were considered, we found that the optimal ratio of cysteamine to capture probe 2 was 1:1.

To ensure the method time-saving, the effect of incubation time of GNPs with thiols on ECL intensity was extensively investigated. Figure 2C shows the ECL calibration curves obtained upon different incubation time (2, 4, 6, 8 and 10 h) of GNPs with cysteamine and capture probe 2. As we can see from Figure 2C, ECL intensity of the ECL nanoprobes increases as the incubation time extends. However, when the time comes to 6 h, the current

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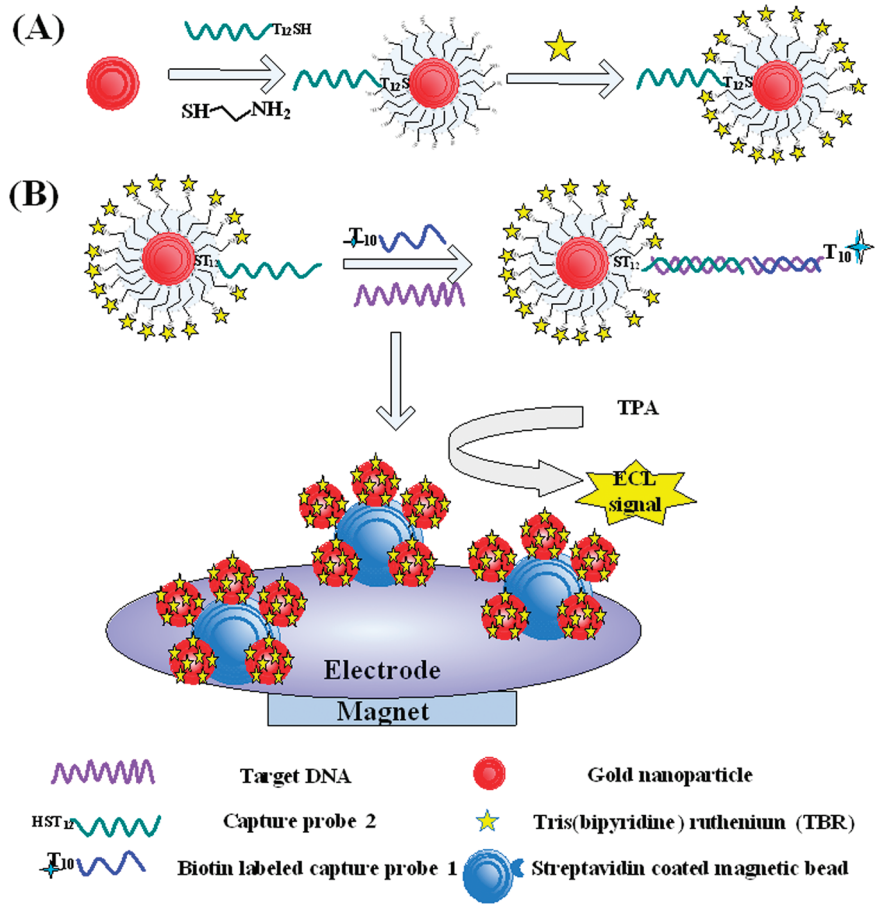

Figure 1. The cysteamine−GNP biobar-code assay: (A) ECL nanoprobe preparation and (B) nanoparticle-based amplification scheme.

signal begins to level off, suggesting that the binding sites of GNPs are replaced by thiols absolutely, so the optimal incubation time is 6 h. In general, the approach presented here reduces the time of loading barcodes on GNPs from 46 to 16 h and eliminates the salt-aging step compared to the conventional assay.

In our initial study, 5 μL of optimal ECL nanoprobes and 5 μL of 1 μM capture probe 1 were mixed in a total reaction volume of 100 μL of phosphate buffer saline (0.01 M, pH 7.4) containing target DNA. The system was allowed to stand at 65 °C for 7 min, then placed on an orbital shaker and incubated at 28 °C for 1 h to hybridize. The products of hybridization were incubated with 15 μL (10 μg/μL) of streptavidin coated MMPs with gentle stirring for 20 min. Application of a magnetic field pulled complexes of MMPs-target-ECL nanoprobe to the wall of the reaction tube in a matter of seconds. The complexes were washed two times with hybridized buffer to effectively remove excess ECL nanoprobes that bound to the MMPs through nonspecific adsorption. The sandwich complexes dissolved by 100 μL of TPA buffer were added to the reaction cell, then captured, and temporarily immobilized on the working electrode by a magnet under it. The photon signal was then measured, when a voltage of 1.25 V was applied.

Figure 3 shows results of ECL assays obtained upon different concentrations of target DNA toward 100 fM. A control experiment, carried out by mixing MMPs with capture probe 1 and ECL nanoprobes in the absence of target DNA, clearly demonstrates that little unspecific binding occurs. It is obvious that ECL intensity increases with an increasing target concentration compared to the control and the target DNA could be quantitatively measured over a large concentration variation from 100 fM to 100 pM. A plateau effect reaches above 100 pM target concentration, which may be due to exhaust of ECL nanoprobes. To define the limit of detection, a cutoff value is calculated based on eq 1

$$V_{\text{cutoff}} = V_{\text{control}} + 3V_{\text{stdev(con)}} \quad (1)$$

where the V_{control} is the average light emission from the control and $V_{\text{stdev(con)}}$ represents the standard deviation of the ECL reading from the control samples. According to this formula, the cutoff was set at 285 counts/s. Note that the signal of 100 fM can still be clearly identified from the threshold value, thus we estimate the limit of DNA detection is 100 fM. To investigate accuracy of the proposed assay, relative standard deviation (RSD) was determined by measuring ECL signal of 100 and 1 pM target DNA with six replicates, RSD values for both concentrations were less than 7%.

The specificity of the sensor was determined by challenging it with single-base mismatches (SM) in the pure buffer. To test the selectivity of this system, the assay was carried out in two pure solutions, each containing either 100 pM concentration of perfect matches (PM) or SM (Figure 4). Control experiments were carried out in the absence of any oligonucleotides. As expected, the ECL signal of the sensor has little changes after treatment with SM compared to the control. The ECL intensity obtained in the presence of PM is much higher than that of the ECL intensity

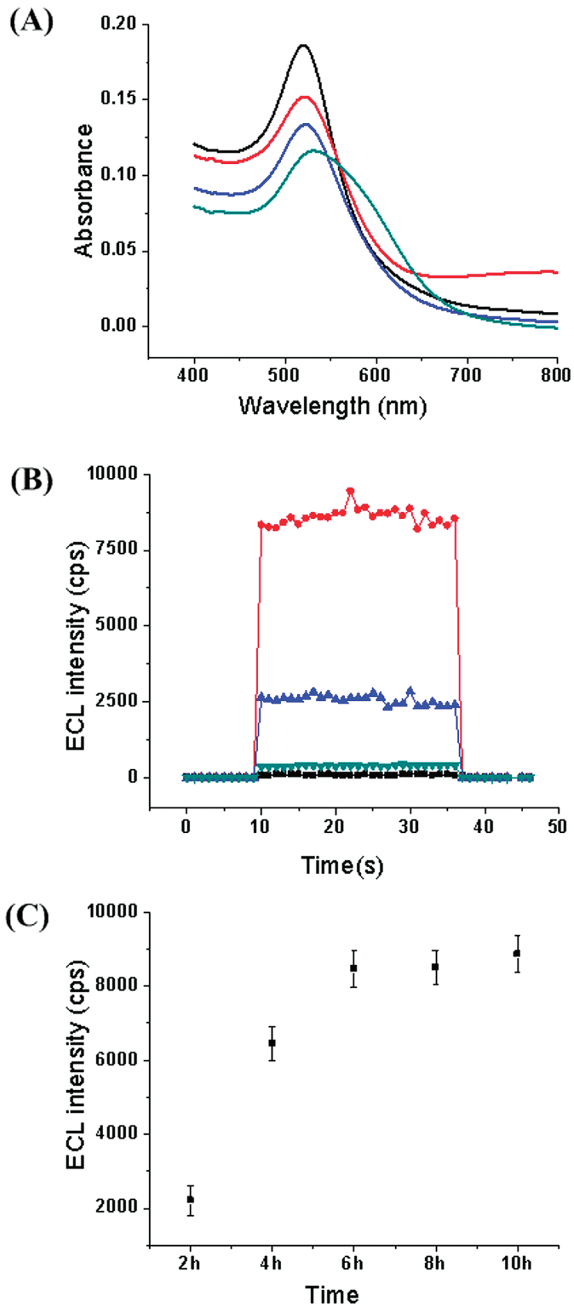

Figure 2. (A) The absorbance spectra of unlabeled GNPs (black) and the prepared ECL nanoprobes. The ratios of cysteamine-to-capture probe 2 are 1:1 (red), 10:1 (blue), and 100:1 (cyan), respectively, Peaks are emerged at 519, 520, 522, and 532 nm, from top to bottom, respectively. (B) ECL intensities from TPA (black) and the resulting ECL nanoprobes according to the different cysteamine-to-capture probe 2 ratios. The ratios are 1:1 (red), 10:1 (blue), and 100:1 (cyan), respectively. (C) Effect of the time on label efficiency of the ECL nanoprobe.

obtained in the presence of the SM. Accordingly, the sensor is highly selective that allows one to differentiate SM.

In an attempt to test the applicability of this DNA sensor for detection in real samples, we employed diluted human serum samples (1:10). Control experiments were carried out in the human serum without any oligonucleotides. Figure 4 shows that there is little discernible signal when a SM is present compared to the control. Importantly, we are able to observe ECL signals corresponding to the presence of target DNA that is comparable

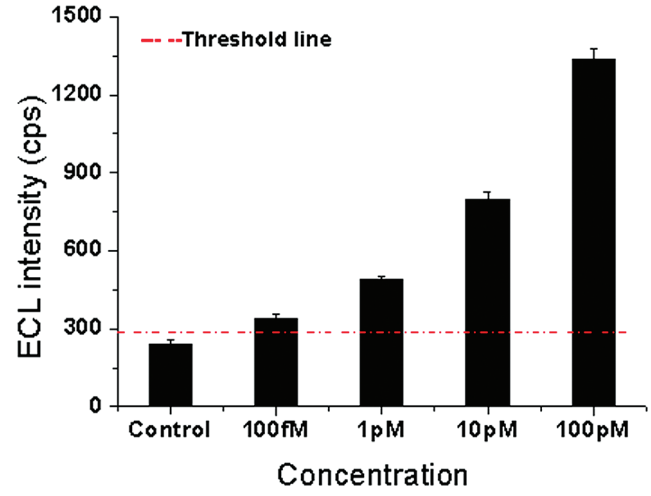

Figure 3. ECL intensities obtained upon hybridization of different concentrations of target DNA.

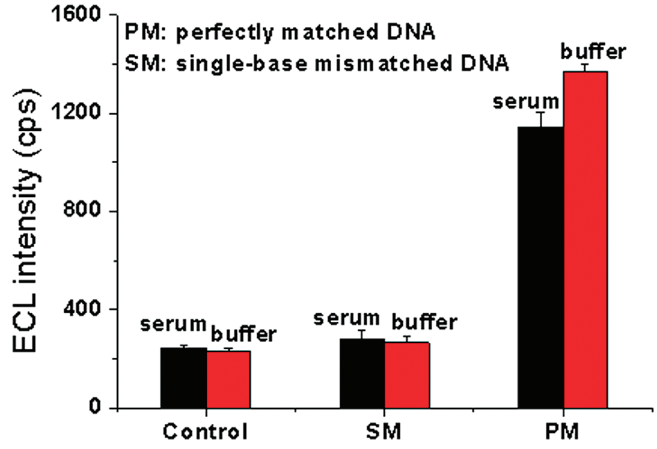

Figure 4. Histograms for ECL intensities at different conditions. From left to right: a human serum sample, a pure buffer sample, 100 pM single-base mismatches in the human serum, 100 pM single-base mismatches in the pure buffer, 100 pM perfect mismatches in the human serum, and 100 pM perfect mismatches in the pure buffer.

to that obtained in pure DNA solutions. We believe the ability to detect SM in human serum would therefore offer an approach for diagnosis applications.

Compared with other biobarcode assays, our new system has the following advantages. First, it eliminates lengthy salt-aging steps, in which the high concentrated salt may lead the GNPs with irreversible aggregation. Second, the employing of cysteamine tends to reduce resistances adsorbed onto GNPs and avoid steric congestion around hybridizing sequences, thereby enhancing sensitivity. Third, as an advantage of using cysteamine rather than costly dual labeling oligonucleotides enabled a reduction in assay costs. Finally, on the basis of previous work, we take advantage of streptavidin-coated MMPs to directly separate target complexes without the release and collection of barcodes, realizing rapid and simple detection. What is more, we employ a magnetic field but not chemical action to gather target sandwiches onto the electrode surface, ensuring the reutilization of the electrode.

To conclude, we have successfully developed an ECL biobarcode assay via introduction of a novel ECL nanoprobe. As

evidenced by our research, this assay allows us to determine oligonucleotides down to 100 fM and exhibits a significant selectivity for SM even in blood serum. The sensitivity of this proposed ECL biobarcode assay for DNA detection is nearly 1 order of magnitude larger than that of these biobarcode assays based on TBR-labeled oligonucleotides,[24,25] fluorescence detection,[36] and electrochemical detection.[37] The sensitivity is also comparable to the recently developed biobarcode assay based on electrochemical detection of dissolved CdS nanoparticles.[14] Although the sensitivity is not as good as the conventional biobarcode assay (from femtomolar to zeptomolar DNA samples),[6,38] which requires a lengthy salt-aging step, release, and immobilization of biobarcode DNA probes for quantification, the current biobarcode version possesses its intrinsic merits, such as simplicity of operation, time-saving, and low cost. Moreover, this method provides a promising platform for utilizing small molecules to fabricate cost-effective, simple, robust, and reusable DNA sensors. Further improvements in the LOD should be possible if larger GNPs can be used.

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