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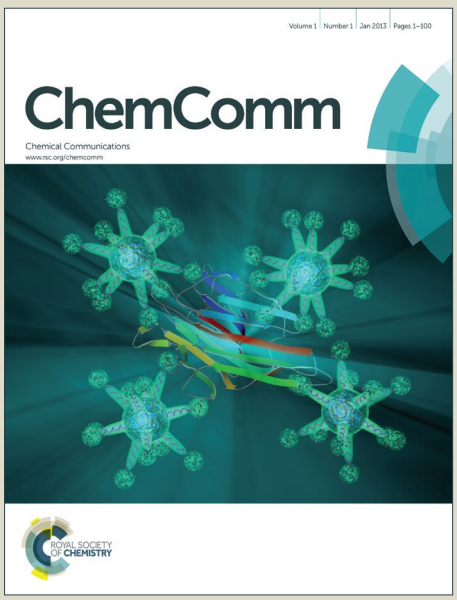

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The determination of DNA methyltransferase activity by quenching of tris(2, 2'-bipyridine)ruthenium electrogenerated chemiluminescence with ferrocene

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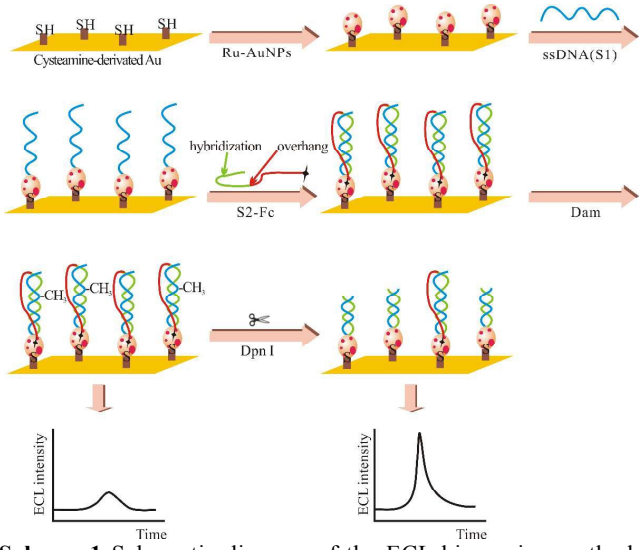
An electrogenerated chemiluminescence (ECL) biosensing method for the determination of DNA methyltransferase activity is developed by the quenching of tris(2, 2'-bipyridine)ruthenium ECL by ferrocene, and it is demonstrated that the ECL biosensing method measures DNA adenine methylation methyltransferase over a dynamic concentration range (0.1 U/mL-100 U/mL) with an extremely low detection limit of 0.03 U/mL, using gold nanoparticles and a quenching ECL signal produced by a chemical quencher such as ferrocene.

DNA methylation is an important epigenetic modification of genes, which impacts the heritable state of gene expression in both prokaryotes and eukaryotes.[1] DNA methylation refers to the process of methyl transfer from the donor S-adenosylmethionine (SAM) to cytosine or adenine in particular short palindromic sequences induced by DNA methyltransferase.[2] Recent studies have revealed that aberrant methylation is a well-recognized hallmark of many diseases.[3] In bacteria, typical sites of methylation include the N_6 position of adenine, the N_4 position of cytosine, or the C_5 position of cytosine, which can protect bacterial DNA against cleavage by restriction enzymes.[4] Therefore, bacterial DNA methyltransferases are essential for bacterial virulence and the viability of several pathogenic bacterial strains.[5,6]

DNA adenine methyltransferase (Dam) is a stand-alone enzyme that methylates GATC sites, which ubiquitously exists in prokaryotic organisms. Dam can convert adenine into *N*6-methyladenine (m^6A) and play several key roles in bacterial processes, including DNA mismatch repair, DNA replication, and regulation of gene expression.[7,8] Because mammals do not methylate DNA at adenine, the bacterial enzymes represent excellent candidates for antibiotic and new therapeutics for diseases development.[9] Therefore, sensitive determination of the activity of Dam and fast screening of its inhibitors have attracted significant attention from both biochemical and clinical researchers.[10-13]

Various techniques have been proposed for the determination of DNA Dam activity such as polymerase chain reaction (PCR),[14] gel electrophoresis, radioactive labeling,[15] fluorescence,[14-19] colorimetry,[20,21] bioluminescence,[22] chemiluminescence,[23] and electrochemical methods.[24-27] Among them, the ECL method has attracted considerable attention and exhibited unique advantages (such as high sensitivity, rapidity, easy controllability, and wide dynamic range) for the specific detection of DNA methylation.[28] Despite these advantages, only a few workers have reported utilizing ECL for Dam activity determination.[29,30] However, due to the high ECL background based on enzyme-linkage reactions and nanoparticle amplification applied in those works,[29,30] the sensitivity and linear range of those biosensors were limited and unsatisfied. So it is necessary to develop novel methods for the determination of Dam with higher sensitvity, especially for point-of-care applications. As a powerful analytical method for the determination of specific quencher species or other substances, the quenching of $Ru(bpy)_3^{2+}$ ECL by different quenchers has attracted great attention.[28,31,32]. Cao et al. reported an efficient and stable quenching of tris(2, 2'-bipyridine)ruthenium(II) ($Ru(bpy)_3^{2+}$) ECL by ferrocene molecule at a gold electrode for the detection of DNA hybridization.[33]



Scheme 1 Schematic diagram of the ECL biosensing method for the determination of DNA methyltransferase activity.

Motivated by the above observation, we present here an ECL biosensor for the highly sensitive determination of DNA methyltransferase activity based on enzyme-linkage reactions incorporated with gold nanoparticles (AuNPs) and ECL emitting species. The aim of this work is to augment the sensitivity of the ECL method for the determination of DNA methyltransferase activity. A diagram of this method for the determination of DNA methyltransferase activity is shown in Scheme 1. To design the biosensor, two kinds of single strand DNA (ssDNA) were used (each 10 μM). The ECL biosensing electrode was fabricated by self-assembling 5'-thiol modified 15-mer ssDNA (S1) contains sequence fragments of 5'-GATC-3' on the surface of a $Ru(bpy)_3^{2+}$–AuNPs composites modified gold electrode, and then hybridized with ssDNA-Fc (S2-Fc) composed of a hybridization sequence (blue) and an

overhang component (red) to form duplex DNA (dsDNA). Adenine (A) nucleotides has high affinity for gold surfaces.[34,35] The designed poly-adenine (polyA) overhang DNA sequence containing the ferrocenyl moiety tag was easily attached to the surface of the $Ru(bpy)_3^{2+}$−AuNPs composites modified gold electrode. In the absence of Dam and SAM, a notably weak ECL signal was observed due to the high ECL quenching efficiency of ferrocene on $Ru(bpy)_3^{2+}$. When Dam and SAM were introduced, all adenine residues within 5'-GATC-3' of dsDNA on the biosensing electrode were methylated. After the methylated biosensing electrode was treated by Dpn I endonuclease, the methylated adenines were cleaved, which causes the S2 with a polyA overhang DNA sequence containing the ferrocenyl moiety tag to be selectively cut off and released. These cleaved fragments dissociate into the bulk solution, allowing the ferrocenyl moiety to move away from the $Ru(bpy)_3^{2+}$−AuNPs composites modified gold electrode surface, thus leading to an increase in the ECL signal. The ECL intensity of the ECL biosensing electrode is related to the methylation level and Dam activity in a fixed concentration Dpn I endonuclease.

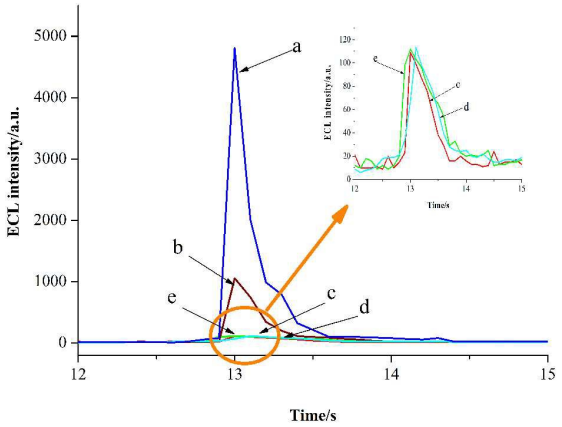


Fig.1 ECL profiles of the different electrodes. S2&S1/MCH/$Ru(bpy)_3^{2+}$-AuNPs/cysteamine modified gold electrode methylated by 100 U/mL Dam and then cleaved with 50 U/mL Dpn I (a), S2&S1/MCH/$Ru(bpy)_3^{2+}$-AuNPs/cysteamine modified gold electrode methylated by 0.5 U/mL Dam and then cleaved with 50 U/mL Dpn I (b), S2&S1/MCH/$Ru(bpy)_3^{2+}$-AuNPs/cysteamine modified gold electrode cleaved with 50 U/mL Dpn I (c), S2&S1/MCH/$Ru(bpy)_3^{2+}$-AuNPs/cysteamine modified gold electrode (d), S3&S1/MCH/$Ru(bpy)_3^{2+}$-AuNPs/cysteamine modified gold electrode methylated by 100 U/mL Dam and then cleaved with 50 U/mL Dpn I (e). The ECL measurement was performed at an applied potential of +0.9 V in 0.10 M PBS (pH 7.4) containing 20 mM TPA.

The morphology and structure of $Ru(bpy)_3^{2+}$−AuNPs composites were characterized by scanning electron microscopy (SEM) and energy dispersive spectrometry (EDS). The results in Fig.S1 show that the $Ru(bpy)_3^{2+}$−AuNPs composites were well synthesized and can be used for further applications in the ECL study. The procedures for the fabrication of DNA sensor were characterized by electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV). These results in Fig.S2 showed that the S1 was immobilized on the gold electrode and the S2/S1 dsDNA was formed and methylated by Dam and cleaved by a methylation-sensitive restriction endonuclease. Fig. 1 demonstrates the corresponding ECL profiles of the differently modified electrodes. Line d in Fig. 1 is the ECL emission of the S2&S1/MCH/$Ru(bpy)_3^{2+}$-AuNPs/cysteamine modified gold electrode, which shows a significantly higher efficiency in quenching the ECL intensity. After the S2&S1/MCH/$Ru(bpy)_3^{2+}$-AuNPs/cysteamine modified gold electrode (unmethylated) is treated with Dpn I (line c), the ECL intensity is the same as S2&S1/MCH/$Ru(bpy)_3^{2+}$-AuNPs/cysteamine modified gold electrode (line d). This indicates Dpn I can not cleave unmethylated sites. When the S2&S1/MCH/$Ru(bpy)_3^{2+}$-AuNPs/cysteamine modified gold electrodes are methylated at the site of 5'-GATC-3' by Dam (100 U/mL) and the treatment of Dpn I endonuclease (line a), the recovered ECL intensity is significantly higher than that for the unmethylated dsDNA (line d), which is attributed to the fact that the S2 having a polyA overhang DNA sequence containing the ferrocenyl moiety tag is efficiently removed from the surface of $Ru(bpy)_3^{2+}$−AuNPs composites modified gold electrode by the cleavage of Dpn I endonuclease at the site of 5'-GAmTC-3'. The above experimental results prove that a sensing interface was effectively constructed according to principle scheme and successfully applied in the determinaton of Dam activity, thereby confirming the feasibility of the biosensor.

One-base mismatched synthetic S3 was selected to evaluate the cleavage specialty of Dpn I endonuclease. It can be seen that the ECL intensity (line e) remained almost invariant compared with lines c and d presented in Fig. 1, attributed to the fact that S1&S3 structure does not contain a specific recognition sequence (5'-GATC-3') of the endonuclease. It was demonstrated that Dpn I can selectively digest 5'-GAmTC-3' of the endonuclease and can distinguish even one-base mismatched DNA. All these results prove that the biosensor presented here has good selectivity. Furthermore, the presented approach can be also applied to monitor the process of methylation and to quantify the methylation level catalyzed by Dam (Fig. S3A). Dependence of the ECL intensity on the methylation time of the S2&S1 was inspected in the range of 5 min to 120 min. The result shown in Fig. S3A is evidence that the longer the treatment time for the Dam, the more methylated adenine that is generated. Nevertheless, when the methylation time is prolonged, the increased rate of ECL intensity gradually decreases, indicating that the methylation catalyzed by Dam is slowed. This phenomenon is probably due to the consumption of the substrates. The methylation is finished within 2 h since the ECL intensity levels out after that time.

SAM acts as the methyl group donor and is thus consumed in this assay. The concentration of SAM therefore has been optimized in this experiment (Fig. S3B). The result shows that the ECL intensity was increased by changing the concentration of SAM from 10 to 160 μM. The signal tended to a relative maximum when the concentration of the SAM exceeded 160 μM. Therefore, 160 μM was chosen as the optimum concentration for SAM in the subsequent experiments.

Under the optimum conditions, the interaction model was further used to analyze the activity of methyltransferase with different concentrations of Dam. The ECL responses to different concentrations of Dam are illustrated in Fig. 2A, the ECL intensity increases with an increase of the activity of Dam in the range of 0.1 U/mL to 100 U/mL. The linear regression equation was *S*=1532.4+1556.1 lgC (unit of C is U/mL) with a regression coefficient of 0.9954 (Fig. 2B). A comparison of performance of different methods for the Dam assay is shown in Table S1. The linear range of the developed method is much wider than that of previously reported Dam assays.[23,29] The detection limitation for Dam in this work was calculated to be 0.03 U/mL (S/N=3), which is lower than those of most reported assays. The relative standard deviation (RSD) for the determination of 20 U/mL Dam at one electrode for seven times was 3.5%. In addition, the RSD for the determination of 20 U/mL Dam at seven different electrodes from the same batch was 3.8%. These suggest the good reproducibility of the ECL biosensors. The evaluation of selectivity of this biosensing method for Dam activity was performed by examining M.Sss I methyltransferase (Fig. S4).

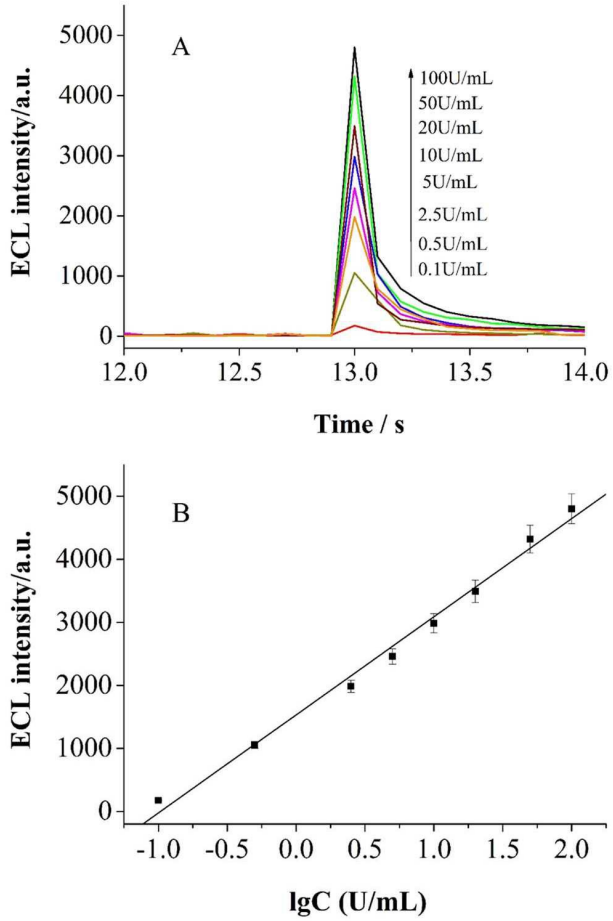


Fig. 2 (A) ECL responses obtained at the ECL biosensor with different activities of Dam. Before the ECL intensity was recorded, the S1&S2 hybrids were methylated by Dam for 2 h and then cleaved by Dpn I for 2 h. (B) Linear relationship between ECL intensity and the the concentration of Dam. The illustrated error bars represent the standard deviation of five repetitive measurements at each concentration.

M.SssI methyltransferase can methylate all cytosine residues within the doublestranded dinucleotide recognition sequence of 5'-CG-3'.[36,37] The results show that a significant increase in ECL is observed in the presence of Dam but no distinct ECL signal is observed in the presence of M.SssI methyltransferase, suggesting the high selectivity of the proposed biosensing method. Therefore, excellent sensitivity, good reproducibility, high selectivity and relatively short assay time (240 min) of the ECL method was obtained.

To further demonstrate the potential application of this method in screening the inhibitor of methyltransferase, the influence of drugs on Dam activity has been investigated by using gentamycin (broad-spectrum antibiotic) and 5-fluorouracil (anti-cancer drug) as two model direct inhibitors. As shown in Fig. S5, the relative activity of Dam decreased with an increasing concentration of gentamycin and 5-fluorouracil. This suggests that the inhibition of the genomic DNA methylation by gentamycin and 5-fluorouracil is remarkably dose-dependent. When trace gentamycin as low as 0.5 μM was introduced into methyltransferase mixture, the relative activity of Dam decreased about 15%. The calculated IC_{50} for gentamycin and benzylpenicillin were 1.0 and 90 μM, respectively. The result indicates that our proposed method is promising for evaluating the capability of the inhibitors.

In conclusion, we have proposed a new ECL method for the highly sensitive determination of DNA methyltransferase activity based on enzyme-linkage reactions incorporated with AuNPs and the quenching of ECL signals produced by a chemical quencher such as ferrocene. The ECL biosensing method measures Dam over a dynamic concentration range (0.1 U/mL-100 U/mL) with an extremely low detection limit of 0.03 U/mL. Furthermore, the method can be also used for evaluating and screening the inhibitors of methyltransferase, which may assist in the discovery of anticancer drugs. It can be extended for the determination of other targets that display DNA methyltransferase activity, and the combination of the proposed approach with an ECL commercial bioanalyzer will be applied in point-of-care testing of other methyltransferases.

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Notes and references

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