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Picomolar level profiling of the methylation status of the p53 tumor suppressor gene by a label-free electrochemical biosensor†

Po Wang,[ab] Hai Wu,[a] Zong Dai*[a] and Xiaoyong Zou*[a]

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An efficient electrochemical approach is developed for ultrasensitive profiling of the methylation status of the p53 tumor suppressor gene based on a label-free biosensor in combination with bisulfite conversion.

DNA methylation is critically associated with DNA replication and repair, genetic imprinting, X chromosome inactivation, and regulation of gene expression.[1–4] The p53 tumor suppressor gene (p53 gene) is one of the most frequently methylated genes of the human genome. When promoter CpG islands in the p53 gene are aberrantly methylated, their downstream genes are found to be consistently silenced,[5] which is regarded as a hallmark of cancer.[6,7] The development of new molecular approaches for the detection of p53 gene methylation can provide a powerful tool for early cancer diagnosis as well as insight into fundamental mechanisms of epigenetic regulation of genetic information.

Several traditional methods have been developed to evaluate genomic DNA methylation, such as methylation-specific polymerase chain reaction (PCR),[8] restriction endonuclease cleavage assay,[9] and methylation-sensitive single nucleotide primer extension.[10] These methods are effective in studying DNA methylation patterns, however, complicated operations, expensive instruments, or radioactive labeling are usually involved.[7,11]

Electrochemical techniques are promising for the analysis of DNA methylation due to their advantages of convenience, rapidity, low cost and ease of miniaturization.[12,13] Okamoto's group pioneered the electrochemical investigation of DNA methylation by selective labeling of 5-methylcytosine (mC) *via* osmium complexation.[14] Niwa *et al.* firstly realized the direct electrochemical detection of mC and cytosine (C) using a nanocarbon film electrode,[12,15] which provided a straightforward approach for exploring DNA methylation. According to the principle of complementary base pairing, we proposed an effective method for the assessment of the C methylation level in a double-stranded DNA sample.[16] Recently, Topkaya *et al.* developed a genosensor for the detection of hypermethylation of the glutathione *S*-transferase P1 gene based on the oxidation of guanine.[17] Most of the current electrochemical approaches are global assays that provide overall levels of C methylation, the realization of gene-specific methylation analysis is a considerable challenge due to the difficulty in the recognition of specific methylation loci in a gene sequence.

Taking advantage of the discriminative capability of *Hpa*II restriction enzyme, the methylation status of a specific CpG site was successfully identified by Cai *et al.*[18,19] This method is rapid and convenient except for the need for labeling procedures. Recently, we proposed a method for quantification of DNA methylation information according to the specific interaction between methylene blue and guanine residues.[20] The method implements the detection of gene-specific methylation without labeling procedures, however, it is limited to the analysis of a single methylation site with the 5′-CC̲GG-3′ sequence.

Selective base conversion with bisulfite is a potential method to probe the C methylation status in DNA.[11] Bisulfite treatment converts unmethylated C into uracil (U) and leaves methylated C intact.[21] The methylation information of C is translated into base sequence information, which paves the way for the recognition of specific methylation sites in the context of DNA sequence. Peptide nucleic acid (PNA) is a structural DNA analogue in which the negatively charged sugar-phosphate backbone is replaced by a neutral polyamide chain.[22] When the PNA probe is not hybridized, there is no available binding site for the loading of a positively charged indicator due to the absence of anionic phosphate groups, suggesting higher selectivity and sensitivity with a low level of noise from the background signal.

Herein, an innovative electrochemical method is presented for the recognition of p53 gene methylation based on a label-free biosensor in combination with bisulfite conversion. The biosensor was constructed by using neutral PNA as a capture probe and $[Ru(NH_3)_6]^{3+}$ as an electroactive indicator. The mechanism of this strategy is illustrated in Scheme 1. The p53 genes were firstly pretreated with bisulfite, the C in the gene sequence was deaminated and then converted to U, whereas mC remained unchanged due to the extremely low reactivity.[15,23] Because mC and C exhibited identical Watson–Crick base-pair behavior,[15] the methylated p53 gene could be effectively captured by the complementary PNA probe, while the unmethylated p53 gene was not the complementary sequence of the PNA probe. As a consequence,

[a] *School of Chemistry and Chemical Engineering, Sun Yat-Sen University, Guangzhou 510275, P. R. China. E-mail: ceszxy@mail.sysu.edu.cn, daizong@mail.sysu.edu.cn; Fax: +86 20 84112245; Tel: +86 20 84114919*
[b] *School of Chemistry and Chemical Engineering, Jiangsu Normal University, Xuzhou 221116, P. R. China. E-mail: wangpo@mail.ahnu.edu.cn; Fax: +86 516 83536977; Tel: +86 516 83403165*

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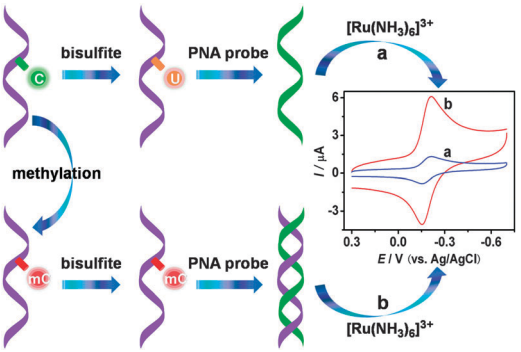

Scheme 1 Schematic illustration of the electrochemical recognition of p53 gene methylation.

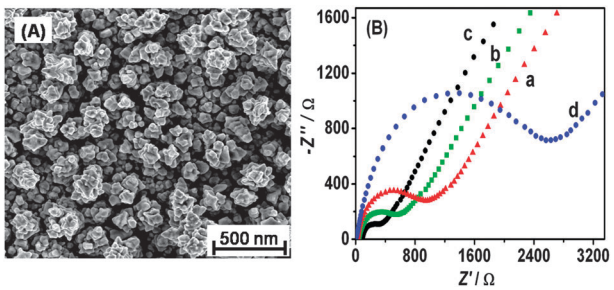

Fig. 1 (A) Field emission scanning electron microscope (FE-SEM) image of the nano-Au/Ch/GCE. (B) EIS of the bare GCE (a), Ch/GCE (b), nano-Au/Ch/GCE (c) and PNA/nano-Au/Ch/GCE (d) in 0.1 M KCl containing 10 mM $[Fe(CN)_6]^{3-/4-}$. Experimental parameters: DC potential, 0.23 V; frequency range, 10^5–0.01 Hz; amplitude, 0.005 V.

the methylation status of the p53 gene was clearly recognized according to the current signal of $[Ru(NH_3)_6]^{3+}$, which can bind electrostatically to the negatively charged backbone of the methylated p53 gene bound to the PNA probe.

As shown in Fig. 1A, the choline ($HOCH_2CH_2N^+(CH_3)_3Cl^-$; Ch) monolayer supported Au nanoparticles film modified glassy carbon electrode (GCE) was designed as the electrode interface for the immobilization of the PNA probe. The Ch was firstly grafted on the surface of the GCE through oxygen atoms (see ESI†, Scheme S1). The purpose of Ch is to provide a stable monolayer with positively charged $-N^+(CH_3)_3$ polar head groups, which could extremely increase the density of edge-plane-like active sites of the GCE for the assembly of Au nanoparticles. As a result, the Au nanoparticles with an average size of about 110 nm were uniformly distributed on the Ch/GCE. They were assembled to form a flowerlike structure, which was suitable for the immobilization of the PNA capture probe. Compared with the bare gold electrode, great enlargement of the effective surface area was achieved for the nano-Au/Ch/GCE, which could load more PNA probes and increase the sensitivity for methylation detection.

The construction process of the biosensor was characterized by electrochemical impedance spectroscopy (EIS). As shown in Fig. 1B, a semicircle of about 950 Ω diameter with an almost straight tail line was presented at the bare GCE (curve a), indicating a diffusional limiting step of the electrochemical process. The electron-transfer resistance (R_{et}) was apparently reduced to 600 Ω at the Ch/GCE (curve b), demonstrating an accelerating effect for the redox reaction of $[Fe(CN)_6]^{3-/4-}$ due to the static gravitation between negatively charged $[Fe(CN)_6]^{3-/4-}$

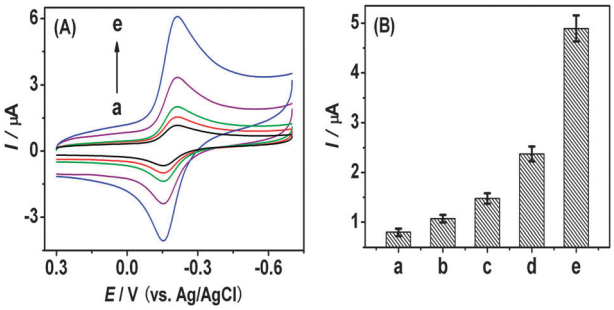

Fig. 2 (A) CVs of PNA/nano-Au/Ch/GCE in 0.1 M pH 7.0 PBS containing 20 μM $[Ru(NH_3)_6]^{3+}$ before (a) and after hybridization with 1.0 μM 5′-CGA GTG GAA GGA AAT TTG CGT GTG GAG TAT TTG GAT GA-3′ (b), 5′-mCGA GTG GAA GGA AAT TTG CGT GTG GAG TAT TTG GAT GA-3′ (c), 5′-CGA GTG GAA GGA AAT TTG mCGT GTG GAG TAT TTG GAT GA-3′ (d), and 5′-mCGA GTG GAA GGA AAT TTG mCGT GTG GAG TAT TTG GAT GA-3′ (e). (B) Corresponding histograms of the reduction peak currents.

and positively charged $-N^+(CH_3)_3$ groups of Ch on the Ch/GCE surface. Moreover, the R_{et} was further reduced to 320 Ω at the nano-Au/Ch/GCE (curve c), which was attributed to the fact that Au nanoparticles can act as excellent electron transfer medium and enhance electron transfer. However, after the immobilization of PNA probes, the R_{et} was significantly increased to 2800 Ω (curve d), indicating that the highly organized PNA probes greatly obstructed the interfacial electron transfer of $[Fe(CN)_6]^{3-/4-}$.

In order to evaluate the analytical performance of the constructed biosensor in recognizing the methylation status of the p53 gene, a series of p53 genes with different methylation status were detected at the PNA/nano-Au/Ch/GCE by cyclic voltammetry (CV) in 0.1 M phosphate buffer solution (PBS) containing the 20 μM $[Ru(NH_3)_6]^{3+}$ indicator. As shown in Fig. 2A, a weak reduction peak located at −0.21 V was found at the PNA/nano-Au/Ch/GCE (curve a), which was attributed to the reduction signal of the $[Ru(NH_3)_6]^{3+}$ indicator. When the PNA probe was hybridized with the unmethylated p53 gene (curve b), the reduction peak of $[Ru(NH_3)_6]^{3+}$ increased only a little compared with that at the PNA/nano-Au/Ch/GCE (curve a), indicating that very weak hybridization occurred between the PNA probe and the unmethylated p53 gene. Actually, after the bisulfite conversion, the unmethylated gene sequence 5′-CGA GTG GAA GGA AAT TTG CGT GTG GAG TAT TTG GAT GA-3′ was converted to 5′-UGA GTG GAA GGA AAT TTG UGT GTG GAG TAT TTG GAT GA-3′, which contains two mismatched bases for the PNA probe, resulting in rather low hybridization efficiency. However, when the p53 gene was methylated gradually, the reduction peak current of $[Ru(NH_3)_6]^{3+}$ increased accordingly (curves c–e). The peak signal of $[Ru(NH_3)_6]^{3+}$ reflected the hybridization efficiency between the p53 gene and the PNA probe, which indirectly revealed the methylation status of the p53 gene. In particular, after the PNA probe was hybridized with the fully methylated p53 gene, remarkable increase in reduction current was obtained (curve e), suggesting the realization of complete hybridization, which accumulated more $[Ru(NH_3)_6]^{3+}$ at the biosensor interface. It was noted that the peak current of $[Ru(NH_3)_6]^{3+}$ was apparently affected by the methylated position of C for single base methylation in the p53 gene. When the methylation occurred in the middle of the sequence (curve d), the

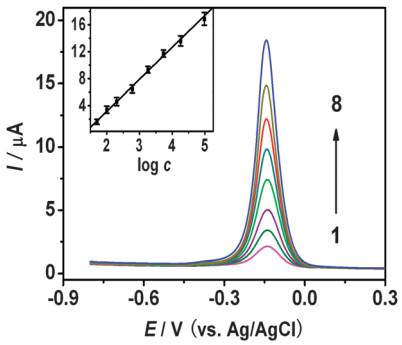

Fig. 3 DPVs of PNA/nano-Au/Ch/GCE in 0.1 M pH 7.0 PBS containing 20 μM $[Ru(NH_3)_6]^{3+}$ after hybridization with the fully methylated p53 gene (5′-mCGA GTG GAA GGA AAT TTG mCGT GTG GAG TAT TTG GAT GA-3′). p53 gene concentrations (from 1 to 8): 50, 100, 200, 600, 1800, 5400, 18 000 and 96 000 pM. Inset is the linear relationship between peak current and the logarithm of concentration.

$[Ru(NH_3)_6]^{3+}$ indicator showed higher peak current than that obtained when the methylation occurred at the top (curve c), indicating the promising capability of the proposed method in the recognition of methylation sites of the p53 gene. This result was attributed to the fact that the mismatch behavior occurred in the center of target DNA exhibited a greater effect on the hybridization efficiency and duplex stability.[24] The resultant histograms of the corresponding reduction peak currents are displayed in Fig. 2B, which provided a more clear resolution of the p53 gene methylation status.

The realization of the electrochemical recognition of the methylation status of the p53 gene is explained as follows: cationic complex $[Ru(NH_3)_6]^{3+}$ did not bind electrostatically to the neutral PNA probe due to the absence of the anionic phosphate group before hybridization. After the PNA–DNA hybrid was formed at the sensor interface, the $[Ru(NH_3)_6]^{3+}$ indicator interacts electrostatically with the negatively charged phosphate groups and is adsorbed on the DNA backbone, giving a clear hybridization detection signal in CV. As for the p53 genes with different methylation status, the C in the gene sequence was converted to U whereas the mC remained unchanged in the process of bisulfite pretreatment. Therefore, the fully methylated p53 gene could be effectively hybridized with the complementary PNA probe, while the unmethylated and partially methylated gene sequences exhibited low hybridization efficiency due to the mismatch behavior. Consequently, the methylation status of C was discriminated according to the hybridization efficiency between the p53 gene and the PNA probe, which can be evaluated from the current signal of the $[Ru(NH_3)_6]^{3+}$ indicator.

Differential pulse voltammetry (DPV) can provide better peak resolution and higher current sensitivity. The quantitative analysis of the fully methylated p53 gene was investigated by DPV instead of CV. As shown in Fig. 3, the reduction peak current of $[Ru(NH_3)_6]^{3+}$ was proportional to the logarithm of the target concentration in a wide range of 50 pM to 96 nM. The linear regression equation is I_{pc} (μA) = 4.9 log$c$ (pM) − 6.7 (n = 10, R = 0.9988). The limit of detection (LOD) was calculated to be 18 pM, which was estimated from the equation LOD = $3S_b/m$, where S_b is the standard deviation of the blank measurements and m is the slope of the calibration curve.

In summary, a pM level methylation analysis of the p53 gene was realized by a label-free electrochemical biosensor in combination with bisulfite conversion. The p53 genes with two different CpG sites and four methylation statuses were successfully recognized according to the reduction current of the $[Ru(NH_3)_6]^{3+}$ indicator. The proposed method exhibited ultrasensitivity for the detection of the fully methylated p53 gene with a LOD of 18 pM, which was about three orders of magnitude lower than previous report.[20] In comparison with the previous electrochemical method to detect DNA methylation sites,[20] the advantages and innovations involved in this work were shown to be sensitive, capable of profiling the methylation status of multiple CpG sites, and suitable for universal analysis of any DNA sequence. We will develop this approach to achieve real sample analysis of the p53 gene obtained from tumor cells, and more related works are in progress.

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