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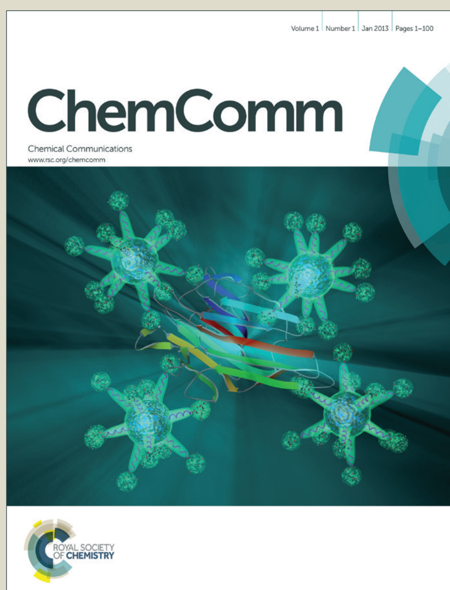

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A novel electrochemical immunosensor for 5-hydroxymethylcytosine quantitative detection in genomic DNA of breast cancer tissue

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A novel electrochemical immunosensor was fabricated for 5-hydroxymethylcytosine (5-hmC) quantitative detection in genomic DNA based on anti-5-hmC antibody, biotin functionalized phos-tag and avidin functionalized alkaline phosphatase. It is demonstrated that the levels of 5-hmC are dramatically reduced in human breast cancer tissue compared with normal tissue.

5-hmC is often considered to be the sixth base of the genome, which can be formed under the catalysis effect of ten-eleven translocation (TET) proteins to 5-methylcytosine (5-mC)[1, 2]. In contrast to 5-mC, the distribution of 5-hmC in mammals is tissue specific and non-random, which is relatively abundant in neuron cells [3], mouse cerebellum [4], and embryonic stem cells[5]. This variance indicates that 5-hmC may play key role in embryonic stem (ES) regulation, the DNA demethylation and epigenetic regulation[1, 6]. Moreover, some studies present that the 5-hmC levels are profoundly reduced in many types of cancer cells[7], which indicates that the change of the expression level of 5-hmC may be used as biomarker for cancer diagnosis. Therefore, the detection of 5-hmC gradually become a hotspot on epigenetics.

In the past decades, various methods have been developed for detection of 5-hmC in genomic DNA, such as single-molecule real-time (SMRT) sequencing [8], liquid chromatography/tandem mass spectrometry (LC/MS-MS) [9], thin layer chromatography (TLC) [10], enzymatic radioactive glycosylation labelling [11], and high-performance liquid chromatography (HPLC) with UV detection [12]. However, though these methods have their own advantages, all of them have limitations still. For example, LC/MS-MS and HPLC require expensive and sophisticated large-scale instrument. SMRT needs additional fluorescent tags. TLC and radioactive glycosylation labeling require radioactive substrates, which is harmful to biological tissue. Our group reported an electrochemical biosensor for 5-hmC detection based on glycosylation and alkaline phosphatase catalytic signal amplification [13]. However, as a proof of concept investigation, this method wasn't applied for detection of 5-hmC in genomic DNA. Therefore, it is still necessary to develop rapid and simple method for detection of 5-hmC in genomic DNA.

Recently, electrochemical immunosensor has gained growing attention since it possesses the advantage the high sensitivity, simple instrument and remarkable specificity. Therefore, electrochemical immunosensor has been applied for the determination of various analytes, such as IgG1 [14], prostate-specific antigen [15] and carcinoembryonic antigen [16]. Our group also fabricated some electrochemical immunosensors for assay of DNA methyltransferase activity [17] and detection of subgroup J avian leukosis virus [18]. However, there is no report with respect to electrochemical immunosensor for 5-hmC detection in the genomic DNA.

Phos-tag is a functional molecule that binds specifically phosphate group. It was widely used for separation of phosphoisotypes of large proteins [19] and detection of phosphorylation of protein [20]. Our group also reported a new electrochemical method for detection of protein kinase activity based on phos-tag [21]. Because phos-tag could specifically recognize the phosphate group of 5-methyl-2'-deoxycytidine 5'-triphosphate (5-hm-dCTP), it shows great potential for detection of 5-hmC as link unit.

In this work, we fabricated a novel electrochemical immunosensor for 5-hmC detection in genomic DNA of breast cancer tissue based on anti-5-hmC antibody, biotin functionalized phos-tag (phos-tag-biotin) and avidin functionalized alkaline phosphatise (adivin-ALP),where anti-5-hmC antibody was selected as 5-hmC recognition unit, phos-tag-biotin was used as link unit, and avidin-ALP was used as enzymatic signal amplification unit. The schematic diagram of the immunosensor was shown in Scheme 1 and the electrode modification process was characterized by electrochemical impedance spectroscopy (EIS, Fig. S1). After graphene-perylenetetracarboxylic acid (GR-PTCA) was activated by

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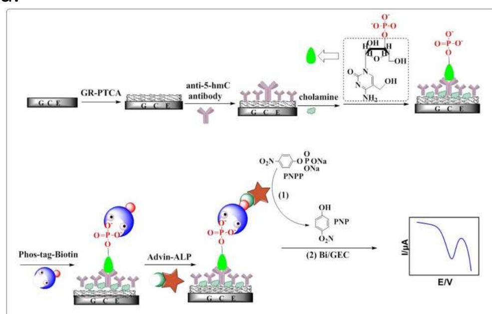
EDC/NHS, anti-5-hmC antibody could be captured on the surface of GR-PTCA functionalized GEC electrode (Ab/GR-PTCA/GCE) through the formation of amido bond. Then 5-hydroxymethyl-2'-deoxycytidine-5'-triphosphate (5-hm-dCTP) could be further assembled on the electrode surface by immuno-reaction between 5-hmC and anti-5-hmC antibody. Afterwards, the phos-tag-biotin could be used as link unit between 5-hm-dCTP and avidin-ALP through the specific reactions between phos-tag and phosphate group of 5-hm-dCTP as well as biotin-avidin affiliation reaction. Under the catalytic effect of ALP towards *p*-nitrophenyl phosphate (PNPP), *p*-nitrophenol (PNP) was produced as electrochemical activity molecule. The higher concentration of 5-hm-dCTP could lead to the enhanced ALP loading amount, which could improve the amount of produced PNP and further increase the differential pulse voltammetry (DPV) response. However, without the 5-hm-dCTP, avidin-ALP cannot be conjugated on the electrode due to the absence of phos-tag-biotin. Therefore, based on the relationship between the oxidation peak current of PNP and the concentration of 5-hmC, 5-hmC can be detected.



Scheme 1. Schematic illustration of the electrochemical immunosensor fabrication and 5-hmC detection.

To assess the detection feasibility of the electrochemical immunosensor, bismuth modified GEC (Bi/GCE) was used as working electrode for electrochemical detection. The DPV behaviour of Bi/GCE was recorded in the detection solution(10 mM Tris-HCl containing 1 mM $MgCl_2$ and 3 mM PNPP, pH 9.8) after it was incubated with different electrodes for 40 min at 37 °C. As shown in Fig 1, no oxidation peak (curve a) for Bi/GCE in blank detection solution was observed. After detection solution was incubated with Ab/GR-PTCA/GCE (which was incubated with phos-tag-biotin and avidin-ALP in turn), the DPV response for Bi/GCE (curve b) is close to that in bank detection solution. This phenomenon can be explained that no catalytic factor was introduced into the detection buffer due to the absence of 5-hm-dCTP. After the detection solution was incubated by Ab/GR-PTCA/GCE (which was incubated with 5-hm-dCTP, phos-tag-biotin and avidin-ALP in turn), an obvious oxidation peak (curve c) was obtained, indicating that the catalytic factor could be captured on the electrode surface in the presence of 5-hm-dCTP. According to the above results, we can conclude that the developed electrochemical immunosensor could be applied to detect 5-hmC.

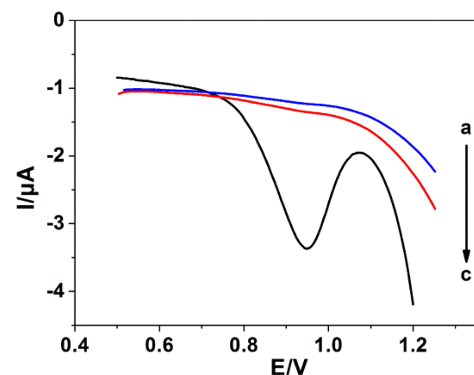


Fig.1. Differential pulse voltammograms of Bi/GCE in 10 mM Tris-HCl (pH 9.8) containing 1 mM $MgCl_2$ and 3 mM PNPP after the solution was treated with different electrodes. (a) Blank detection buffer, (b) Ab/GR-PTCA/GCE was incubated with phos-tag-biotin and avidin-ALP in turn, (c) Ab/GR-PTCA/GCE was incubated successively with 5-hm-dCTP, phos-tag-biotin and avidin-ALP.

To improve the detection sensitivity, 5-hm-dCTP reaction time and avidin-ALP concentration were optimized. With increasing 5-hm-dCTP reaction time from 0 to 120 min, the DPV response increased obviously (Fig S2). However, the DPV response increased slowly when further prolonging the reaction time to 120 min, which indicates that 5-hm-dCTP loading amount tend to saturate. Therefore, 120 min was selected as optimal 5-hm-dCTP reaction time. ALP is used as catalytic factor in the immunosensor, which plays a key role in this work. Thus the concentration of avidin-ALP was also optimized in this work. The DPV response increased obviously with extending the concentration of avidin-ALP from 5 to 50 μg·mL^{-1} (Fig. S3). However, the DPV response increased slowly when the concentrations of avidin-ALP exceed 50 μg·mL^{-1}, indicating avidin-ALP loading amount tend to saturate. Therefore, 50 μg·mL^{-1} was chosen as the optimal avidin-ALP concentration.

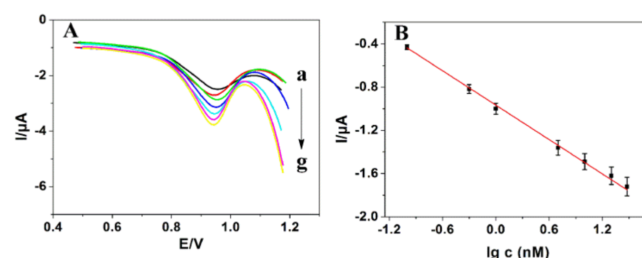


Fig. 2. (A) DPV response of Bi/GCE for different concentrations of 5-hm-dCTP. a-g, 0.1, 0.5, 1, 5, 10, 20, 30 nM. (B) The linear relationship between the DPV response and the logarithm value of the concentration of 5-hm-dCTP.

Under optimized experimental conditions, different concentrations of 5-hm-dCTP were used to prepare immunosensor and the DPV response was recorded. As shown in Fig. 2A, the DPV response increased with increasing the 5-hm-dCTP concentration from 0.1 to 30 nM. Moreover, the logarithm value of DPV current showed a linear relationship with 5-hm-dCTP concentration. As illustrated in Fig. 2B, the

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linear regression equation was expressed as I_{pc} (μA) = -0.531log*c* (nM) – 0.977 (*R* = 0.9981). And the detection limit was estimated to be 0.032 nM (S/N = 3).

5-hmC plays a crucial role in tumor size, tumor invasion, lymph node metastasis and cancer-related death [22]. A study conducted by Yang *et al*. demonstrated that 5-hmC is substantially reduced in multiple human tumors[23], which could be used as biomarker for cancer diagnosis. Thus, repaid detection of 5-hmC levels in biological samples is significant for the diagnosis of cancer. In this work, the electrochemical immunosensor was also applied for 5-hmC quantitative detection in genomic DNA of breast cancer tissue. As shown in Fig. 3A, independent of the three groups of genomic DNA (a, b, c) without DNase I digestion show only band in electrophoresis images, respectively. However, after the genomic DNA was degraded by DNase I, non-migrating bands (d, e, and f) were observed in electrophoresis images. The electrophoresis result suggests that genome DNA was successfully degraded by DNase I. On the basis of the standard calibration curve obtained previously, we can calculate the levels of 5-hmC in different samples. As shown in Fig.3B, the levels of 5-hmC is dramatically reduced in human breast cancer tissues (which were obtained from two cancer patients respectively) compared with normal breast tissue.

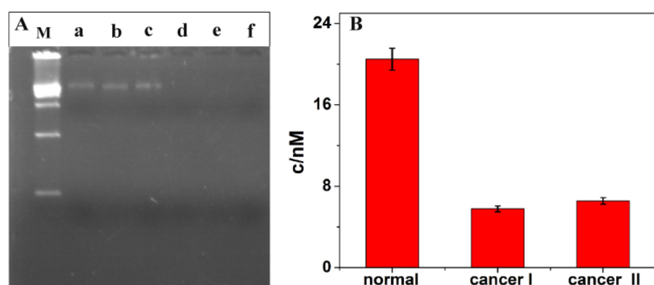

Fig. 3. (A) Gel electrophoresis of genomic DNA extracted from different tissues (a, d: normal breast tissues, b, c, e, f: breast cancer tissues). Lane M: markers, lane a-c: the genomic DNA are without DNase I digestion, lane e-f: the genomic DNA are digested with DNase I. (B) The levels of 5-hmC in different samples.

In summary, we successfully fabricated a novel electrochemical immunosensor for 5-hmC detection in genomic DNA of breast cancer tissue. Based on anti-5-hmC antibody as 5-hmC recognition unit, phos-tag-biotin as link unit, and avidin-ALP as enzymatic signal amplification unit, the electrochemical immunosensor showed excellent detection sensitivity and selectivity. Furthermore, we demonstrated that the levels of 5-hmC are dramatically reduced in human breast cancer tissue when compared with normal breast tissue. The electrochemical immunosensor opens a new perspective in the field of ultrasensitive 5-hmC detection.

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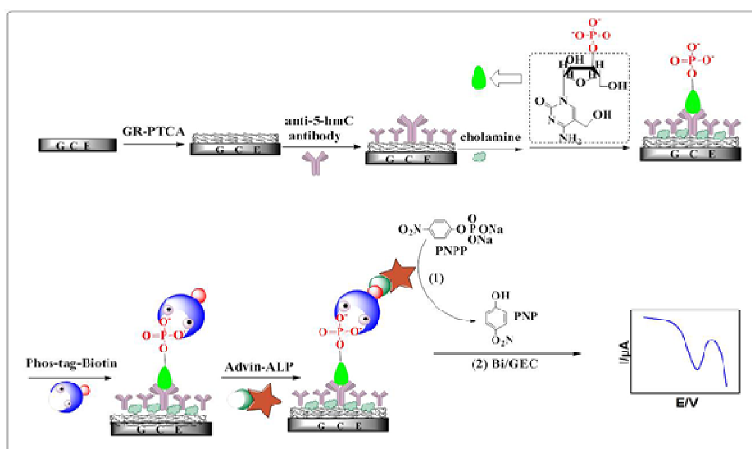

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