A new strategy for methylated DNA detection based on photoelectrochemical immunosensor using Bi$_2$S$_3$ nanorods, methyl bonding domain protein and anti-his tag antibody

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**Abstract**

In this work, we fabricated a novel photoelectrochemical immunosensor for assay of DNA methylation, where Bi$_2$S$_3$ nanorods were used as photoelectric conversion material, MBD1 protein (a kind of methyl bonding domain protein) was used as DNA methylation recognizing unit, anti-his tag antibody was used to further inhibit the photocurrent and increase the detection sensitivity. The results demonstrated that Bi$_2$S$_3$ possessed excellent photoelectron property. The detection conditions, such as Bi$_2$S$_3$ concentration, MBD1 protein concentration, incubation time of MBD1 protein, antibody concentration and antibody incubation time, were optimized. Under optimal experimental conditions, the photocurrent variation was proportional to the logarithm of methylated target DNA concentration from $10^{-9}$ to $10^{-13}$ M with detection limit of $3.5 \times 10^{-14}$ M ($S/N=3$). Moreover, the immunosensor presented high detection specificity, even distinguishing single-base mismatched sequence.

**Keywords:** DNA methylation, Photoelectrochemical immunosensor, Bi$_2$S$_3$ nanomaterial, Methyl bonding domain protein, Anti-his tag antibody

1. Introduction

DNA methylation is one of the most important epigenetic events both in prokaryotes and eukaryotes that plays important role in regulation of gene expression, eukaryotes development, and cellular differentiation (Suzuki and Bird, 2008). Aberrant DNA methylation can be associated with inactivation of tumor suppressor genes, thus leading to carcinogenesis (Palivath et al., 2010; Vaissière et al., 2009). Therefore, the assay and detection of DNA methylation with high sensitivity and specificity is important with respect to early diagnosis of genetic diseases associated with aberrant DNA methylation. To achieve this aim, various methods have been proposed, such as bisulfite coupled with fluorescence detection (Cao and Zhang, 2012; Wan et al., 2007), DNA methyltransferase–restriction endonuclease-based fluorescence (Zhao et al., 2013) or electrochemical detection (Wang et al., 2013), polymerase chain reaction (PCR)-based technique (Lyko et al., 2000), colorimetric approach (Ge et al., 2012a), high-performance liquid chromatography (HPLC) (Lopez Torres et al., 2011) and liquid chromatography–tandem mass spectrometry (Quinlivan and Gregory, 2008), etc. For bisulfite assay, it needs long time to convert cytosine to uracil, which might cause intense DNA degradation. DNA methyltransferase–restriction endonuclease assay suffers from the inactivation of enzyme. For chromatography techniques, the detection instruments are expensive, the diction process are laborious and complicated. More importantly, the professional operators are needed. Therefore, it is still a challenge to develop new method for DNA methylation detection for overcoming the above drawbacks.

Photoelectrochemical biosensor is a kind of newly developed and promising analytical method for biological assay. Up to now, many biological molecules have been detected by photoelectrochemical biosensor, such as dihydroxycitramine adenine dinucleotide (Wang et al., 2009a), α-fetoprotein (Wang et al., 2009b), SMMC-7721 human hepatoma carcinoma cells (Qian et al., 2010), acetylcholinesterase (Huang et al., 2013), and mouse IgG (Zhao et al., 2012). More importantly, photoelectrochemistry (PEC) have been manifested its promising application in DNA analysis (Golub et al., 2012; Lu et al., 2008; Zhang et al., 2011, 2013; Zhao et al., 2011). Therefore, we think that photoelectrochemical biosensor might be an excellent detection platform for DNA methylation assay. However, one of the important influencing factors for photoelectrochemical detection is the photo-to-current conversion efficiency of photoelectric material. Binary metal chalcogenides have drawn extensive attention as they are an important class of semiconductors with high photoelectrochemical activity. Among various chalcogenide materials, bismuth sulfide (Bi$_2$S$_3$) is known to be an attractive material for photoelectrochemistry application as it has a reasonably low band gap (1.3–1.7 eV), an absorption coefficient in the order of $10^4$–$10^5$ cm$^{-1}$ in the visible wavelength range, and a reasonable incident photon to electron absorption.
conversion efficiency (about 5%) (Becerra et al., 2011; Peter et al., 2003; Tahir et al., 2010; Wang et al., 2009c), which making it widely applied in visible-wavelength photodetectors (Konstantatos et al., 2008; Li et al., 2012), photoelectrochemical (PEC) solar cells (Suarez et al., 1998), thermoelectric cooling devices (Chen et al., 1997) and electrochemical hydrogen storage (Zhang et al., 2006). Though Bi$_2$S$_3$ has attractive photoelectrochemical properties (Cheng et al., 2012; Lu et al., 2012; Xiao et al., 2012), to the best of our knowledge, there are no reports on its application in fabricating photoelectrochemical biosensors for analysis of DNA methylation.

Recently, as a kind of DNA methylation recognition proteins, methyl-CpG-binding domain (MBD) proteins have attracted much more attentions on its application in assay of DNA methylation (Serre et al., 2010; Yin et al., 2013; Yu et al., 2010). There are at least five mammalian MBD proteins, namely MeCP2, MBD1, MBD2, and MBD3 for transcriptional repression, and MBD4 (also known as MED1) for mismatch repair as a thymine glycosylase (Fujita et al., 2003). Up to now, methyl-binding proteins have been engineered by fusing a purification tag to monomeric or polymeric MBD1 methyl-binding domains. And these engineered MBD proteins demonstrate significant specificity of CpG recognition, suggesting that MBDS could be used as specific analytical tools for detection of DNA CpG methylation (Jørgensen et al., 2006; Porter et al., 2007; Stains et al., 2006).

In the present work, we developed a novel photoelectrochemical method for analysis of DNA methylation based on Bi$_2$S$_3$ nanorods, recombinant MBD1 protein labeled with 6×His and anti-his tag antibody. As shown in Scheme 1, after CpG methylated probe DNA S1 hybridized with its complementary and methylated CpG target DNA S2, the MBD1 protein was captured on the electrode surface through the specific interaction between MBD1 protein and symmetrical cytosine methylation in CpG region of double-stranded DNA. Then, through the immunoreaction between His tag at the end of MBD1 protein and anti-his tag antibody, anti-his tag antibody was further captured on the electrode surface. Based on the immobilization of MBD1 protein and antibody, the photoelectrochemical response decreased significantly, which was used to detect methylated DNA.

2. Experimental section

2.1. Reagents and Instruments

Bismuth nitrate (Bi(NO$_3$)$_3$), sodium sulfide (Na$_2$S), chloroauric acid (HAuCl$_4$), ascorbic acid (AA), glycol, urea, trisodium citrate, tris(hydroxymethyl)aminomethane (Tris) and tris(2-carboxyethyl)phosphine hydrochloride (TECP, 98%) were purchased from Aladdin (Shanghai, China). 3-Mercaptopropionic acid (MPA) was obtained from Alfa Aesar (Heysham, Lancashire, UK). Anti-his tag antibody was supplied by MBL International Corporation (MA, USA). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, USA). 0.5% (w/v) BSA was prepared with 10 mM phosphate buffered solution (PBS). AuNPs were synthesized according to previous report (Liu and Lu, 2006). Recombinant plasmids pET30(b+)-1xMBD1 were received from Adrian Bird’s lab (Edinburgh, UK). The MBD1 protein expression was performed according to previous report (Yu et al., 2010).

The synthetic oligonucleotides were purchased from Shanghai Sangon Biotechnology Co. (Shanghai, China) and used without further purification. The base sequences of oligonucleotides are as follows: thiol-capped methylated probe DNA S1, 5′-SH-(CH$_2$)$_6$-TAG TGT GAT GTC ACC TAG TTG ACC TTC C(CH$_3$)GG AT-3′; methylated target DNA S2, 5′-ATC C(CH$_3$)GG AAG GTC AAC TAG GTG ACA TCA CAC TA-3′; one-base mismatched DNA S3, 5′-ATC CTG AAG GTC AAC TAG GTG ACA TCA CAC TA-3′; unmethylated probe DNA S4, 5′-SH-(CH$_2$)$_6$-TAG TGT GAT GTC ACC TAG TTG ACC TTC CGG AT-3′; unmethylated target DNA S5, 5′-ATC CCG AAG GTC ACC TAG GTG ACA TCA CAC TA-3′. The synthesized oligonucleotides were diluted in TE buffer (Containing 10 mM Tris–HCl and 1 mM EDTA, pH 8.0) to desired stock concentrations and stored at −20°C according to the manufacturer’s instructions. Double distilled deionized water was used throughout the experiments.
X-ray diffraction (XRD) pattern was obtained with a D8 Advance X-ray diffractometer using Cu Kα radiation (λ=0.15416 Å, Bruck, Germany). SEM image was observed with a Hitachi S-4800 scanning electron microscope (SEM, Hitachi, Japan). PEC measurements were performed with a home-built PEC system. A 500 W Xe lamp equipped with a monochromator was used as the irradiation source to produce the monochromatic light (20 mW/cm²). The photocurrent was recorded on a CHI832A electrochemical workstation (CHI instruments, Austin, USA) with a three-electrode system in PBS (0.1 M, pH 7.0) containing 0.1 M AA, which was deaerated by nitrogen for 30 min before experiments and then kept in a nitrogen atmosphere for the entire experimental process.

2.2. Synthesis of Bi₂S₃ nanorods

Bi₂S₃ nanorods was synthesized according to the previous report with some mirror modifications (Ge et al., 2012b). In brief, 1.82 g Bi(NO₃)₃·5H₂O (0.00375 mol) was firstly added into 25 ml glycol, followed by stirring for 20 min. The obtained solution was noted as solution A. Meanwhile, 1.35 g Na₂S (0.005625 mol) was introduced into 30 mL redistilled deionized water and stirred for 10 min. The obtained solution was noted as solution B. Then the solution B was dripped slowly into the solution A. A large number of black suspended matters produced in the mixed solution, in which 1.35 g urea (0.032 mol) and 20 ml redistilled deionized water were added. The mixed solution was then transferred into a Teflon-lined stainless steel autoclave (120 mL capacity), sealed and maintained at 120 ℃ for 24 h. The resultant black solid product was filtered, washed with redistilled deionized water and dried in air.

2.3. Fabrication of photoelectrochemical immunosensor

The ITO slices were cleaned by ultrasonication in 1 M NaOH solution solved in 50% ethanol for 30 min, followed by acetone for 30 min. Then, the electrodes were washed by double distilled deionized water for three times and dried at 120 ℃ for 2 h.

A certain amount of Bi₂S₃ powder was dispersed ultrasonically in double distilled deionized water, and then 40 μL of the suspension was dropped onto a piece of ITO slice with fixed area of 0.196 cm². After drying in air, the electrode was rinsed with double distilled deionized water for three times. The obtained electrode was named as Bi₂S₃/ITO. Then, 20 μL AuNPs suspension was casted onto Bi₂S₃/ITO electrode surface and dried in air (noted as AuNPs/Bi₂S₃/ITO). Subsequently, the fabricated electrode was incubated with 20 μL 0.1 μM methylated probe DNA S1 for 12 h under humid conditions, followed by rinsed with 10 mM Tris–HCl (pH 7.4) for three times. The electrode was named as S1/AuNPs/Bi₂S₃/ITO. After that, the electrode was further incubated with 10 μL Tris–HCl (10 mM, pH 7.4) containing 3 mM MPA for 1 h to eliminate the un-immobilized probe DNA S1 and hold a good orientation of the immobilized probe DNA S1 for its good recognition ability. The hybridization experiment was performed by dripping 20 μL DNA hybridization buffer containing 0.1 μM of methylated target DNA S2 on the electrode surface and the hybridization process was kept at 37 ℃ for 2 h under humid conditions. After that, the electrode was rinsed three times with 10 mM Tris–HCl (pH 7.4) to remove the un-hybridized target DNA and dried with nitrogen blowing. The obtained electrode was noted as S1–S2/AuNPs/Bi₂S₃/ITO.

Afterwards, 20 μL MBD1 protein (0.1 mg/mL) was dropped on the electrode surface and incubated for 1.5 h under humid conditions at 37 ℃. The obtained electrode (noted as MBD1/S1–S2/AuNPs/Bi₂S₃/ITO) was rinsed with 10 mM Tris–HCl (pH 7.4). After blocking with 0.5% BSA for 30 min, 20 μL anti-his tag antibody (10 μg/mL) was dropped on the electrode surface and incubated for 80 min at 37 ℃ under humid conditions. The electrode (noted as Antibody/MBD1/S1–S2/AuNPs/Bi₂S₃/ITO) was then rinsed three times with 10 mM Tris–HCl (pH 7.4).

3. Results and discussion

3.1. Characterization of Bi₂S₃

The synthesized Bi₂S₃ powder was first characterized by XRD and the corresponding peaks was shown in Fig. 1A. The position and intensity of all the peaks were in good agreement with the reported powder diffraction file (17-0320). And from Fig. 1A, the Bi₂S₃ powder could also be identified as the orthorhombic phase. The morphology of the synthesized Bi₂S₃ powder was investigated by SEM and displayed in Fig. 1B. It showed that the Bi₂S₃ powder mostly consisted of nanorods with diameter in the nanorange and length of a few micrometers.

3.2. EIS and photoelectrochemical characterization of the immunosensor

The fabrication process of the photoelectrochemical immunosensor was characterized by electrochemical impedance spectroscopy (EIS) and photoelectrochemical measurements. Fig. 2A showed the Nyquist plot of ITO electrodes with different modification process using Fe(CN)₆³⁻/⁴⁻ as redox probe. Compared with the bare ITO electrode (curve a), the diameter of the high frequency semicircle of Bi₂S₃/ITO (curve b) decreased, indicating a reduced electron transfer resistance (Rₑₑ), which was caused by the immobilization of semiconductor material of Bi₂S₃. Then, the Rₑₑ value further decreased when AuNPs were modified on Bi₂S₃/ITO electrode (curve c). It could be attributed to the good conductivity of AuNPs, which facilitated the electron transfer. After assembling
methylated probe S1, an increased semi-circle at high frequency region was observed (curve d). The increase in $R_{et}$ could be ascribed to the immobilization of negatively charged phosphoric acid backbone of methylated probe DNA, which repelled the negatively charged Fe(CN)$_6^{3-}$ inhibit the electron transfer. After the probe hybridized with methylated target DNA (curve e), the $R_{et}$ value further increased. With the subsequent stepwise immobilization of MBD1 protein and anti-his tag antibody, the $R_{et}$ value increased gradually as the experiment process (curve f–g), testifying the successfully assembling of DNA methylation recognizing and signal amplifying element on the electrode surface. The increase of $R_{et}$ was that the nonconductive properties of MBD1 protein and antibody obstructed the electron transfer of the redox probe from solution to electrode surface.

The fabricated immunosensor was also characterized by monitoring the photocurrent. As shown in Fig. 2B, an excellent strong photoelectrochemical signal was observed after Bi$_2$S$_3$ were immobilization on ITO surface (curve a), indicating that Bi$_2$S$_3$ possessed the good photoelectrical property. After immobilization of AuNPs on Bi$_2$S$_3$/ITO (curve b), the photocurrent decreased. Then, the photocurrent further decreased gradually after methylated probe DNA S1 immobilization (curve c) and hybridization with methylated target DNA S2 (curve d), which might be caused by two possible reasons. (1) The diffusion rate of electron donor, AA, was decreased photocurrent. As expected, the photocurrent continued to decrease when MBD1 protein (curve e) and anti-his tag antibody (curve f) was assembled on the electrode surface successively. This photocurrent decrease could be attributed to the increase in steric hindrance for AA to diffuse to the electrode surface due to the formation of methylated DNA-MBD1 protein conjugate and the immunocomplex of MBD1 protein-anti-his tag antibody. In addition, one can conclude that this successive photocurrent decrease was caused by the capture of MBD1 protein and anti-his tag antibody, which indicated that the symmetrical cytosine methylation in double-strand DNA could be recognized by MBD1 protein, and anti-his tag antibody could be further captured on the electrode through the immunoreaction between 6 × his-tag at the end of MBD1 protein and antibody. Based on these analysis, the developed photoelectrochemical immunosensor could be applied to detect DNA methylation.

3.3. Optimization of experimental conditions

The effect of the Bi$_2$S$_3$ concentration on the photocurrent responses for 10 pM methylated target DNA S2 was examined at the ranges from 0.1 to 8 mg/mL. As shown in Fig. 3A (see Supporting information), it was found that the photocurrent responses increased with the increase of concentration up to 4 mg/mL. Then, the decrease in photocurrent responses was observed, which might be caused by the increased thickness of Bi$_2$S$_3$ film, decreasing the transfer rate of photoelectron and enhancing the undesirable recombination. Thus, 4 mg/mL was adopted as the optimal concentration in this work.

The effect of the MBD1 protein concentration on the photocurrent responses for 10 nM methylated target DNA S2 was also examined at the ranges from 1 to 80 μg/mL (Fig. S1B in Supporting information). The photocurrent response increased with the MBD1 protein concentration increasing from 1 to 50 μg/mL, then the photocurrent response tended to level off, indicating that almost all the methylation site had been recognized by MBD1 protein and no more protein could be further modified on the electrode surface. Therefore, 50 μg/mL MBD1 protein was used throughout the work. Also, the incubation time was an important parameter for immunoassay. As presenting in Fig. S1C (see Supporting information), the photocurrent response increased with the incubation time extended from 10 to 100 min, then a plateau was obtained with further extending the incubation time. Considering the experiment efficiency, 100 min was selected as the optimal incubation time for MBD1 protein. Similarly, the effect of the concentration (Fig. S1D in Supporting information) and incubation time (Fig. S1E in Supporting information) of anti-his tag antibody on the photocurrent response was also investigated and optimized. The optimal conditions were 120 μg/mL and 70 min for concentration and incubation time, respectively.

3.4. Calibration curve of the immunosensor

The photocurrent response was directly related to the concentration of methylated target DNA S2. Hence, the concentration of

Fig. 2. A) Nyquist plot of different electrodes in 5 mM Fe(CN)$_6^{3-}$-solution containing 0.1 M KCl. (a) ITO, (b) Bi$_2$S$_3$/ITO, (c) AuNPs/Bi$_2$S$_3$/ITO, (d) ssDNA/AuNPs/Bi$_2$S$_3$/ITO, (e) dsDNA/AuNPs/Bi$_2$S$_3$/ITO, (f) MBD1/dsDNA/AuNPs/Bi$_2$S$_3$/ITO, and (g) Antibody/MBD1/dsDNA/AuNPs/Bi$_2$S$_3$/ITO. B) Photocurrent response of different electrodes in 0.1 M PBS (pH 7.4) containing 0.1 M ascorbic acid (λ = 420 nm). (a) Bi$_2$S$_3$/ITO, (b) AuNPs/Bi$_2$S$_3$/ITO, (c) ssDNA/AuNPs/Bi$_2$S$_3$/ITO, (d) dsDNA/AuNPs/Bi$_2$S$_3$/ITO, (e) MBD1/dsDNA/AuNPs/Bi$_2$S$_3$/ITO, and (f) Antibody/MBD1/dsDNA/AuNPs/Bi$_2$S$_3$/ITO.
methylated target DNA S2 could be detected by monitoring the photocurrent signal of immunosensor. Fig. 3A presented the photocurrent after the methylated probe DNA S1 hybridized with various concentration of methylated target DNA S2. The photocurrent decreased with increasing target DNA S2 concentration. As shown in Fig. 3B, the photocurrent variation was proportional to the logarithm of target DNA S2 concentration from $10^{-13}$ to $10^{-9}$ M. The regression equation was $\Delta I (\mu A) = -2.76 \log c (M) - 46.49$ ($R=0.9988$), where $\Delta I = I_0 - I_f$ and $I$ were the photocurrents of S1/AuNPs/Bi$_2$S$_3$/ITO and Antibody/MBD1/S1 hybridate with the same process. When not in use, these electrodes were stored in 10 mM Tris–HCl (pH 7.4) containing 0.01% NaN$_3$ at $4^\circ$C in a refrigerator. Every seven days, one of these electrodes was taken out for photocurrent measurement. The photocurrent $\Delta I$ was observed at S1–S2 hybrid electrode. Because only S1–S2 hybrid has the symmetrical cytosine methylation site in double-strand DNA sequence of 5′-CCGG-3′, it could be recognized by MBD1 protein and anti-his tag antibody could be further captured on the electrode surface, resulting in the lowest photocurrent. For S1–S5, S4955 and S4–S2 hybrids, though they can completely hybridized on the electrode surface, however, all of them do not have the symmetrical cytosine methylation site in double-strand DNA sequence of 5′-CCGG-3′. Consequently, they cannot be recognized by MBD1 protein, in other words, MBD1 protein cannot be assembled on the electrode surface to decrease the photocurrent. For S1–S3 hybrid, there is a mismatched base in recognition site of MBD 1 protein, which not only makes MBD 1 protein failed to be captured on the electrode surface, but also leads to the imperfect hybridization of S1–S3, presenting a highest photocurrent. According to the above analysis, the developed assay has excellent detection selectivity and specificity.

The reproducibility of the developed photoelectrochemical immunoassay was evaluated by intra-assay and inter-assay coefficients of variation (CV). The intra-assay was estimated by assaying methylated target DNA S2 concentration for five times, which resulted in 6.8% and 7.5% at DNA S2 concentration of $10^{-7}$ and $10^{-10}$ M. The inter-assay precision was estimated by detecting $10^{-10}$ M DNA S2 in one sample using five immunosensors fabricated with the same process. The average of coefficient was 7.28%, indicating a satisfactory reproducibility.

The stability of the immunosensor was also investigated. Five Antibody/MBD1/S1–S2/AuNPs/Bi$_2$S$_3$/ITO electrodes were fabricated with the same process. When not in use, these electrodes were stored in 10 mM Tris–HCl (pH 7.4) containing 0.01% NaN$_3$ at $4^\circ$C in a refrigerator. Every seven days, one of these electrodes was taken out for photocurrent measurement. The photocurrent decreased progressively with the storage time extending from 7 to 28 days. The electrode could retain 90.28% of its original photocurrent, implying good acceptable stability.

4. Conclusion

In summary, we confirmed that Bi$_2$S$_3$ could be successfully applied in photoelectrochemical sensor as photoelectric conversion material using visible light as the light source. Based on it, we provided a sensitively photoelectrochemical strategy for detection of CpG methylated DNA using MeCP2 protein as recognition unit for methylated DNA and anti-his tag antibody as signal amplification unit for the first time. The relatively low detection limit of...
3.5 \times 10^{-14} \text{ M and a broad dynamic range of } 10^{-9} - 10^{-13} \text{ M were achieved. The developed assay is highly selective and sensitive, and without bisulfite treatment. This Bi$_2$S$_3$-based photoelectrochemical sensor strategy opens a new perspective in the field of ultrasensitive DNA methylation analysis.}

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**Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.07.040.

**References**


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