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Short Communication

Electrochemical biosensor for DNA demethylase detection based on demethylation triggered endonuclease BstUI and Exonuclease III digestion

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ABSTRACT

Herein, an electrochemical biosensor was fabricated for DNA demethylase detection based on DNA demethylation triggered endonuclease BstUI and Exonuclease III digestion. After the double-strand DNA was demethylated, it can be further digested by BstUI and formed a blunt end at the electrode surface. Then, the remained fragment of DNA–DNA duplex was further cleaved by exonuclease III and led to increased electrochemical signal. Based on this detection strategy, the biosensor showed high sensitivity with low detection limit of 0.15 ng/mL. Moreover, the developed method also presented high selectivity and acceptable reproducibility. This work provides a novel detection platform for DNA demethylase detection.

1. Introduction

DNA methylation is an important epigenetic modification and plays crucial role in differential control of gene expression (Siegfried and Cedar, 1997). It is catalyzed by DNA methyltransferase (MTase), which can transfer one methyl group from the donor of *S*-adenosyl-*L*-methionine (SAM) to the C-5 position of cytosine, and produces 5-methylcytosine (5-mC) and *S*-adenosyl-*L*-homocysteine (SAH) (Horton et al., 2006). Due to the correlation of many diseases with the aberrant expression of DNA MTase, DNA methylation and DNA MTase activity have been considered as potential biomarkers for diseases diagnosis. Though DNA methylation has ever been supposed as a kind of stable epigenetic modification, DNA demethylation process also exists, which is catalyzed by DNA demethylase to remove the methyl from 5-mC (Bhattacharya et al., 1999).

As the opposite process of DNA methylation, DNA demethylation also plays vital roles in gene epigenetic regulation and involves in many diseases, such as cancers, imprinting-related diseases, and psychiatric disorders (Suzuki et al., 2006; Tsankova et al. 2007; Wang et al., 2012). Therefore, it is crucial to develop a sensitive method for the assessment of DNA demethylase activity. However, for this assay, the difficulties came from not only the low abundance of DNA demethylase, but also possible intereference, such as some proteins coexisting with DNA demethylase (Lopez-Serra et al., 2006). Unlike the wide researches on assaying the activity of DNA MTase, little work has been done for detecting DNA demethylase. Recently, enzymatic control of plasmonic coupling as a surface enhanced Raman scattering (SERS) nanosensor was fabricated for DNA demethylation detection and DNA demethylase assay (Wang et al., 2012). After the hemimethylated DNA probes were demethylated, the degradation reaction of the probes methylation-sensitive endonuclease Bsh1236I and single-strand selective exonuclease I was triggered, which resulted in the aggregation of gold nanoparticles (AuNPs) and generated strong plasmonic coupling SERS signal in response to DNA demethylation. This work showed the advantages of instrument miniaturization, low background signal, excellent specificity, convenient, and reproducible detection with homogeneous, single-step operation. However, this method suffers from expensive and large-volume instruments for signal detection, which might limit its applicability.

Electrochemical methods have been demonstrated their virtues on DNA methylation detection and DNA MTase activity assay with the advantages of simple operation, easy preparation, low cost, fast response, high sensitivity and selectivity (Deng et al., 2014; He et al., 2011; Li et al., 2012; Liu et al., 2011; Sato et al., 2006; Sato et al., 2012; Su et al., 2012; Wang et al., 2013; Yin et al., 2013a). Thus electrochemical techniques should also be a suitable detection platform for the assay of DNA demethylase activity. Herein, we develop a simple and sensitive electrochemical method for DNA demethylase detection based on its catalytic demethylation activity and demethylation trigged endonuclease BstUI–Exonuclease III

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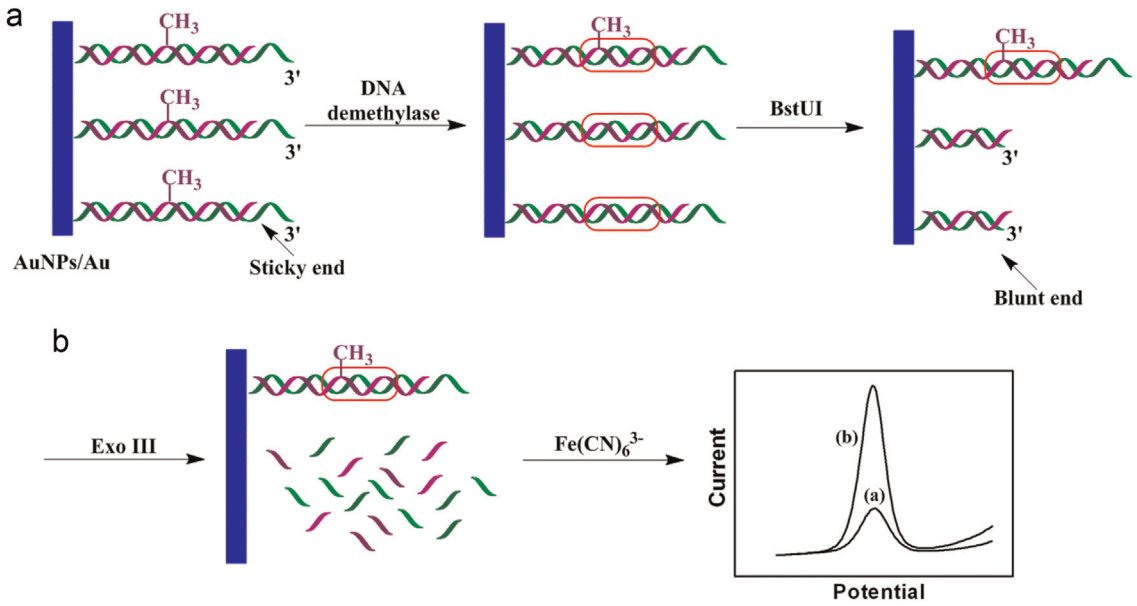

Scheme 1. Illustration of DNA demethylase detection based on electrochemical biosensor.

digestion, where Exonuclease III has been demonstrated its application in DNA detection (Fan et al., 2012; Luo et al., 2013). The detection mechanism is shown in Scheme 1. For improving the electrode effective surface and conductivity, the bare Au electrode is first modified with gold particles (AuPs) through electrochemical deposition under constant potential. Then, the DNA probe S1 is assembled on AuPs/Au surface through the Au–S bond and hybridized with the cytosine-methylated complementary DNA S2, forming hemi-methylated double-strand DNA hybrids, which contain the common recognition sequences (5′-CGCG-3′) for DNA demethylase and endonuclease BstUI (only digesting unmethylated sequence). Without demethylation, the BstUI activity is inhibited due to the cytosine methylation in 5′-CGCG-3′ sequence. Moreover, the formed S1–S2 hybrids contain a 3′-protrusion sticky end, which blocks the digestion activity of Exonuclease III because it has 3′ to 5′ exodeoxyribonuclease activity, which is specific for double-stranded DNA (Henikoff, 1984). Thus, at this state, the S2–S1/AuPs/Au cannot be digested by BstUI–Exonuclease III system, which causes a low electrochemical reduction current due to the stronger electrostatic repulsion effect between the negative charge controlled phosphate backbones of the oligonucleotides and the redox probe of $[Fe(CN)_6]^{3-}$ (Sharifi et al., 2014). However, when the S2–S1 hybrids are demethylated by DNA demethylase, they can be further cleaved by BstUI at the middle of 5′-CG|CG-3′ and form a blunt end at the electrode surface. Afterwards, those residual S2–S1 hybrids can be further digested by Exonuclease III, which will greatly decrease the electrostatic repulsion effect and result in a high electrochemical signal. Based on it, a signal "on" electrochemical method can be developed for DNA demethylase detection.

2. Experimental

2.1. Reagents and instruments

DNA demethylase and MBD4 protein were purchased from Epigentek Group Inc. (Farmingdale, NY, USA). MBD1 and MeCP2 protein were expressed and purified as described in our previous works (Yin et al., 2013a; Yin et al., 2013b). BstUI and Exonuclease III were obtained from New England Biolabs (USA). Tris(hydroxymethyl)aminomethane (Tris), EDTA and tris(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Aladdin (Shanghai, China). All DNA sequences were supplied by Sangon Biotech Co., Ltd. (Shanghai, China). The oligonucleotides sequences were selected randomly and shown as follows. DNA probe S1, 5′-SH–$(CH_2)_6$–TTC ACC TGT GTT TTT TCA TG*C GCG* AGA TCC CCC-3′, hemi-methylated complementary DNA (DNA S2), 5′-TCT $\underline{C}^{m}$*GC G*CA TGA AAA AAC ACA GGT GAA-3′.

The buffer solutions employed in this work are as follows. Probe immobilization buffer, 10 mM Tris–HCl, 1.0 mM EDTA, 1.0 M NaCl and 1.0 mM TCEP (pH 7.4). DNA hybridization buffer, 10 mM Tris–HCl, 1.0 mM EDTA, and 1.0 M NaCl (pH 7.4). DNA demethylase buffer, 10 mM Tris–HCl, 200 mM NaCl, and 10 mM $MgCl_2$ (pH 7.5). Exo III reaction buffer, 10 mM Tris–HCl, 10 mM $MgCl_2$, and 1 mM DTT (pH 7.0). BstUI reaction buffer, 10 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM KCl, and 0.1 mg/mL BSA (pH 8.5). Electrochemistry determination buffer, 10 mM PBS containing 5 mM $[Fe(CN)_6]^{3-}$ and 0.1 M KCl (pH 7.4). Washing buffer, and 10 mM Tris–HCl (pH 7.4).

Differential pulse voltammetry (DPV) was performed at CHI830D electrochemical workstation (CH Instrument, Inc. Austin, USA) in the potential range of −0.2–0.6 V. The parameters of increment potential, 0.004 V; pulse amplitude, 0.05 V; pulse width, 0.05 s; sample width, 0.0167 s; pulse period, 0.2 s; and quiet time, 2 s. The plane Au (d=2 mm, geometric area is 0.0314 cm^2, Gaoss Union®, China) or modified Au electrode was used as the working electrode, saturated calomel electrode as the reference electrode and platinum wire as the counter electrode.

2.2. DNA immobilization and hybridization

The plane gold electrode was firstly polished to mirror-like surface using 0.3 μm alumina slurry, followed with ultrasonic cleaning in double-distilled deionized water and anhydrous alcohol for 3 min. The gold electrode was rinsed with double-distilled deionized water and dried under N_2 blowing. Then the gold electrode was immersed into 3 mM $HAuCl_4$ solution containing 0.1 M KNO_3 and the AuPs were electrochemically deposited on the electrode surface using single-potential mode at −0.2 V for 200 s under stirring. The morphology of this electrode (AuPs/Au) was characterized by SEM (Supplementary materials). Afterwards, the gold electrode was incubated with 5 μL of probe immobilization buffer containing 0.5 μM probe DNA S1 for 12 h at ambient temperature under humid environment. The electrode was rinsed with washing buffer three times and noted as S1/AuPs/Au.

Subsequently, 5 μL of probe immobilization buffer containing 1.5 μM 6-mercapto-1-hexanol was dropped on the S1/AuPs/Au surface and incubated for 1 h to obtain a well-aligned probe monolayer and remove the unspecific adsorbed probe. After rinsed with washing buffer three times, the probe DNA S1 was hybridized with complementary DNA S2 by incubating the S1/AuPs/Au electrode with 5 μL of DNA hybridization buffer containing 0.5 μM DNA S2. The hybridization process was performed for 2 h at 37 °C in a humidified chamber. The obtained electrode (noted as S2–S1/AuPs/Au) was then rinsed three times with washing buffer to remove the un-hybridized target DNA S2.

2.3. DNA demethylation

The S2–S1/AuPs/Au electrode was incubated with 5.0 μL of DNA demethylase buffer containing different concentrations of DNA demethylase for 60 min at 37 °C under humid conditions. Then, the electrode was rinsed with washing buffer three times.

2.4. Digestion by endonuclease BstUI and Exonuclease III

Endonuclease BstUI digestion was performed at 37 °C for 50 min by incubating the demethylated S2–S1/AuPs/Au with 5.0 μL of BstUI reaction buffer containing 20 unit/mL BstUI. After digestion, the electrode was thoroughly rinsed with washing buffer three times and dried with nitrogen. Afterwards, the electrode was immersed with 5.0 μL of Exonuclease III reaction buffer containing 35 unit/mL Exonuclease III for 40 min. Then, the electrode was rinsed with washing buffer three times.

3. Results and discussion

The biosensor characterization and the detection feasibility assay are performed by DPV technique. As shown in Fig. 1A, after AuPs are deposited on the bare Au electrode surface (curve b), the reduction peak current at about 0.2 V increases significantly compared with the Au electrode (curve a), which can be attributed to the increased electrode effective area (the electrochemically active area for Au and AuPs/Au was calculated to be 0.082 and 0.64 cm^2, see Supplementary materials). Then, the reduction current decreases successively after probe DNA S1 assembly (curve c) and hybridization (curve d), which can be explained as the electrostatic repulsion effect between the immobilized oligonucleotides and the redox probe of $[Fe(CN)_6]^{3-}$ (Sharifi et al., 2014). After S2–S1/AuPs/Au is incubated with DNA demethylase, the electrochemical reduction current only increases a little (data not shown), which should be caused by the elimination of methyl at the C-5 location of cytosine. However, the methyl is only a very small group compared with the whole S2–S1 hybrid. So the signal increase caused by the demethylation process is very limited. Afterwards, the reduction current increases greatly when the demethylated electrode is incubated with BstUI (curve e).It can be ascribed to the digestion effect of BstUI towards the recognition sequence of 5′-CGCG-3′ due to the elimination of the obstructive factor of C-5 methylation. The S2–S1 hybrid is cleaved to be two fragments from the middle of 5′-CG|CG-3′ and produces a blunt end, which facilitates further digestion of Exonuclease III, resulting in a strong electrochemical reduction current (curve f).

For proving the vital role of DNA demethylase in the detection strategy, S2–S1/AuPs/Au is directly incubated with BstUI and Exonuclease III successively. Then the DPV response is recorded and shown in Fig. 1B. The electrochemical response (curve b) is close to the response obtained at S2–S1/AuPs/Au (curve a) and lower than that at S2–S1/AuPs/Au after incubation with DNA demethylase, BstUI and Exonuclease III successively (curve c), which

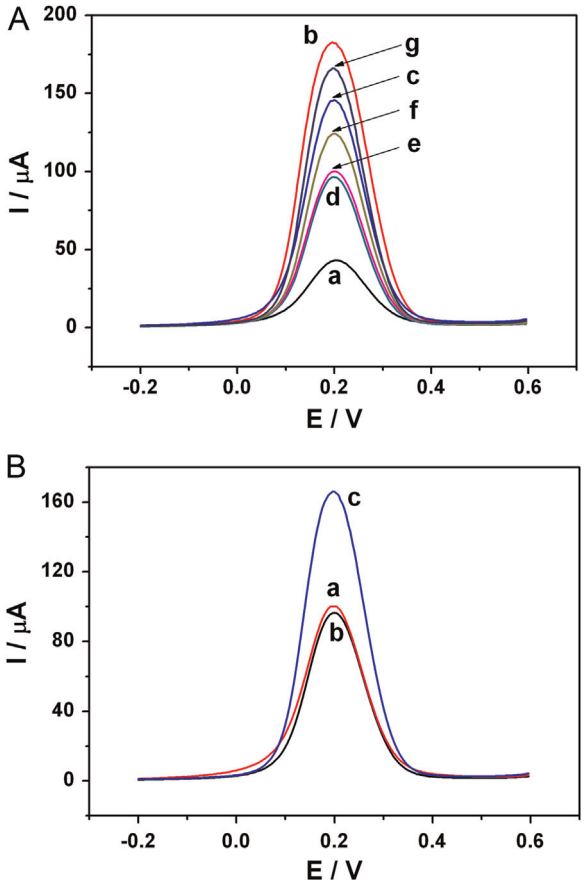

Fig. 1. (A) Differential pulse voltammograms of different electrodes in 10 mM PBS containing 5 mM $[Fe(CN)_6]^{3-}$ (pH 7.4) and 0.1 M KCl. (a) Au, (b) AuPs/Au, (c) S1/AuPs/Au, (d) S2–S1/AuPs/Au, and (e) S2–S1/AuPs/Au was demethylated by 50 ng/mL DNA demethylase. (f) The demethylated electrode was further incubated with 20 unit/mL BstUI. Curve (g) was further incubated with 35 unit/mL Exonuclease III. (B) Differential pulse voltammograms of S2–S1/AuPs/Au in 10 mM PBS containing 5 mM $[Fe(CN)_6]^{3-}$ (pH 7.4) and 0.1 M KCl with different treatment processes. (a) S2–S1/AuPs/Au, (b) S2–S1/AuPs/Au was directly incubated with BstUI and Exonuclease III, and (c) S2–S1/AuPs/Au was incubated with DNA demethylase, BstUI and Exonuclease III successively.

implies that the activities of BstUI and Exonuclease III are inhibited due to the methylated cytosine in the sequence of DNA S2. From these investigations, we can conclude that the developed method can be applied to detect DNA demethylase.

To assess the detection performance of the proposed method under optimal conditions (the detection conditions were optimized as described in Supplementary materials), the differential pulse voltammograms were recorded after the S2–S1/AuPs/Au was first incubated with different concentration of DNA demethylase, and then with 20 unit/mL BstUI and 35 unit/mL Exonuclease III successively. As the concentration of DNA demethylase varied from 0.5 to 100 ng/mL, the electrochemical reduction current increased continually (Fig. 2A), which indicated that the S2–S1 hybrids were gradually cleaved and hydrolyzed by BstUI and Exonuclease III. The plot for the electrochemical reduction current and the concentration of DNA demethylase was illustrated in Fig. 2B. The electrochemical reduction current increased linearly with the logarithm value of demethylase concentration in the range from 0.5 to 70 ng/mL with the linear regression equation of I_{pc} (μA)=34 (±3)log c (ng/mL)+107(±8) (R=0.9927, shown in the inset of Fig. 2B). The detection limit was estimated to be 0.15 ng/mL (S/N=3), which was lower than previous work using electrochemical method (1.3 ng/mL) (Zhou et al., 2014) and closed to at surface enhanced Raman scattering (0.1 ng/mL) (Wang et al., 2012).

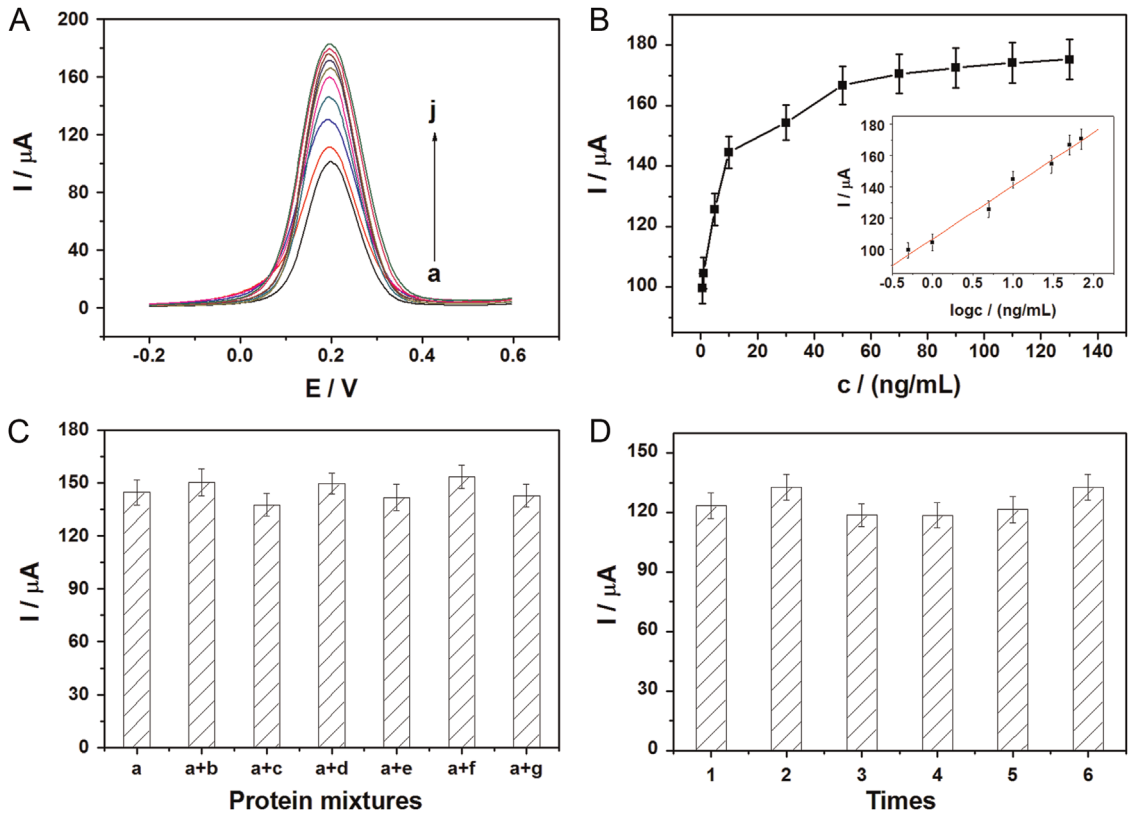

Fig. 2. (A) Differential pulse voltammograms of S2–S1/AuPs/Au after incubated with different concentrations of DNA demethylase (a–j: 0.5, 1, 5, 10, 30, 50, 70, 90, 110, 130 ng/mL), 20 unit/mL BstUI and 35 unit/mL Exonuclease III successively. (B) The plot of the relationship between DNA demethylase concentration and electrochemical response. Inset: Calibration curve. (C) The histogram for the reduction peak current of S2–S1/AuPs/Au after incubated with different protein mixtures. (a) DNA demethylase, (b) MBD1 protein, (c) MBD4 (50 ng/mL), (d) MeCP2 protein, (e) uracil-DNA glycosylase, (f) RNase H, and (g) histone H2A. (D) The histogram for the reduction peak current of the biosensor for six parallel detections.

The detection specificity was evaluated by incubating the S2–S1/AuPs/Au with the complex of DNA demethylase and other possible coexistence proteins, BstUI and Exonuclease III successively. Then, the differential pulse voltammograms were recorded and the reduction peak current (0.2 V potential) was compared. For comparison, the S2–S1/AuPs/Au was also incubated with DNA methylase, BstUI and Exonuclease III successively. As illustrated in Fig. 2C, the electrochemical reduction current for S2–S1/AuPs/Au digested by DNA demethylase (10 ng/mL) was close to those obtained at the same electrode digested by the complex of DNA methylase (10 ng/mL) and other possible coexistence proteins, such as MBD1 protein (50 ng/mL), MBD4 (50 ng/mL), MeCP2 protein (50 ng/mL), uracil-DNA glycosylase (UDG, 50 unit/mL), RNase H (50 unit/mL) and histone H2A (50 ng/mL), indicating that these proteins almost do not interfere DNA demethylase detection. These results also prove the excellent detection specificity of the developed method.

The reproducibility of the fabricated biosensor was testified by detecting 5 ng/mL DNA demethylase with six electrodes prepared independently with the RSD of 5.2% (Fig. 2D, detection process is shown in Supplementary materials). After S2–S1/AuPs/Au was stored for two weeks at 4 °C in refrigerator, it was incubated with DNA demethylase, BstUI and Exonuclease III successively. The electrochemical reduction current remained 91.4% of its original value, implying good stability.

4. Conclusions

In summary, a simple, sensitive and selective electrochemical method was developed for the detection of DNA demethylase based on demethylation triggered BstUI and exonuclease III digestion. Without demethylation, the dsDNA could not be digested by BstUI and Exonuclease III because the BstUI could not recognize the sequence of 5′-C^{m}GCG-3′. As a result, a low electrochemical response was obtained. On the contrary, after demethylation the DNA–DNA hybrids could be cleaved by BstUI and then by Exonuclease III. Hence, a strong electrochemical response was obtained. The developed method presented the advantages of simple operation, high sensitivity and selectivity.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.11.026

References

Bhattacharya, S.K., Ramchandani, S., Cervoni, N., Szyf, M., 1999. Nature 397 (6720), 579–583.

Deng, H., Yang, X., Yeo, S.P.X., Gao, Z., 2014. Anal. Chem. 86 (4), 2117–2123.
Fan, Q., Zhao, J., Li, H., Zhu, L., Li, G., 2012. Biosens. Bioelectron. 33 (1), 211–215.
He, X., Su, J., Wang, Y., Wang, K., Ni, X., Chen, Z., 2011. Biosens. Bioelectron. 28 (1), 298–303.
Henikoff, S., 1984. Gene 28 (3), 351–359.
Horton, J.R., Liebert, K., Bekes, M., Jeltsch, A., Cheng, X., 2006. J. Mol. Biol. 358 (2), 559–570.
Li, W., Wu, P., Zhang, H., Cai, C., 2012. Anal. Chem. 84 (17), 7583–7590.
Liu, S., Wu, P., Li, W., Zhang, H., Cai, C., 2011. Chem. Commun. 47 (10), 2844–2846.
Lopez-Serra, L., Ballestar, E., Fraga, M.F., Alaminos, M., Setien, F., Esteller, M., 2006. Cancer Res. 66 (17), 8342–8346.
Luo, C., Tang, H., Cheng, W., Yan, L., Zhang, D., Ju, H., Ding, S., 2013. Biosens. Bioelectron. 48, 132–137.
Sato, S., Hokazono, K., Irie, T., Ueki, T., Waki, M., Nojima, T., Kondo, H., Takenaka, S., 2006. Anal. Chim. Acta 578 (1), 82–87.
Sato, S., Tsueda, M., Kanezaki, Y., Takenaka, S., 2012. Anal. Chim. Acta 715 (0), 42–48.
Sharifi, E., Salimi, A., Shams, E., Noorbakhsh, A., Amini, M.K., 2014. Biosens. Bioelectron. 56, 313–319.
Siegfried, Z., Cedar, H., 1997. Curr. Biol. 7 (5), R305–R307.
Su, J., He, X., Wang, Y., Wang, K., Chen, Z., Yan, G., 2012. Biosens. Bioelectron. 36 (1), 123–128.
Suzuki, K., Suzuki, I., Leodolter, A., Alonso, S., Horiuchi, S., Yamashita, K., Perucho, M., 2006. Cancer Cell 9 (3), 199–207.
Tsankova, N., Renthal, W., Kumar, A., Nestler, E.J., 2007. Nat. Rev. Neurosci. 8 (5), 355–367.
Wang, Y., He, X., Wang, K., Su, J., Chen, Z., Yan, G., Du, Y., 2013. Biosens. Bioelectron. 41 (0), 238–243.
Wang, Y., Zhang, C.-H., Tang, L.-J., Jiang, J.-H., 2012. Anal. Chem. 84 (20), 8602–8606.
Yin, H., Zhou, Y., Xu, Z., Chen, L., Zhang, D., Ai, S., 2013a. Biosens. Bioelectron. 41, 492–497.
Yin, H., Zhou, Y., Xu, Z., Wang, M., Ai, S., 2013b. Biosens. Bioelectron. 49 (0), 39–45.
Zhou, Y., Li, B., Wang, M., Yang, Z., Yin, H., Ai, S., 2014. Anal. Chim. Acta 840, 28–32.