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Ultraselective homogeneous electrochemical biosensor for DNA species related to oral cancer based on nicking endonuclease assisted target recycling amplification

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Abstract

Traditional electrochemical DNA biosensors need DNA immobilization on the electrode surface, which is tedious and time consuming. In this study, a simple but ultraselective electrochemical DNA biosensor had been designed to determine target DNA species related to oral cancer overexpressed 1 in saliva, which combines the signal amplification of nicking endonuclease assisted target recycling with the immobilization-free electrochemical method. The complementary substrate strand of target DNA species contains a simple asymmetric sequence had been modified with a methylene blue at the 3'terminal firstly, which can not diffuse easily to the negative charged ITO electrode surface due to the abundant negative charges. The present of the target DNA would trigger the formation of double-stranded DNA(dsDNA). Then the nicking endonuclease can recognize the simple asymmetric sequence in the dsDNA and cleave the substrate strand of ds-DNA into two pieces, a long ssDNA and a 2-base ssDNA linked with methylene blue. The short one can diffuse easily to the negative charged ITO electrode surface and results in the enhanced electrochemical response detected. At the same time, the target DNA can dissociate from the dsDNA and trigger the next round of hybridization, cleavage and releasing, which results in the signal amplification. This homogeneous DNA biosensor can detect as low as 0.35 pM (S/N=3) target DNA. Compared with the traditional heterogeneous electrochemical DNA biosensors which is tedious and time consuming due to complex DNA immobilization process, the assay not only owns the merits of simple and high efficiency since performed in a homogeneous solution, but also exhibits high

distinction ability to single-base mismatch, double-bases mismatch, and noncomplementary DNA sequence.

Keywords: homogeneous, immobilization free, electrochemical biosensor, nicking endonuclease signal amplification, ITO electrode.

Oral cancer is one of the most common malignancies leading to death among head and neck squamous cell carcinoma (HNSCC) worldwide[1,2] and which results in more than 11,000 deaths each year in the US alone[3]. Currently, diagnosis in cancer cell lines and diagnosis in biopsy specimens from late invasive and metastatic cancers are the main methods for oral cancer diagnosing. But these methods have some side effects on the human body, such as result in exposure to HIV or hepatitis[4]. Moreover, oral cancer initially are asymptomatic lesions and can not be diagnosed or treated until they reach an advanced stage. In order to reduce death rate from oral cancer, it is necessary to develop some non-invasive early diagnosis method. Recently, with the discovery of cancer-related nucleic acids in oral fluid, oral fluid has become a diagnostic fluid which has many obvious advantages over the blood[5].

Development of efficient methods for highly sensitive and rapid screening of specific DNA sequences is essential to the early diagnosis of serious diseases[6,7]. Oral Cancer Overexpressed 1 (ORAOV1), also called Tumor Amplified and Overexpressed Sequence 1, is a novel gene locating at chromosome band 11q13 in saliva[8,9]. According to the early literature reported, ORAOV1 amplification is significantly associated with larger tumor size[10]. ORAOV1 can act as a novel target and be explored to develop therapeutic strategy in oral cancer management[11]. The electrochemical biosensor has been recognized as a promising tool for DNA detection because of simple equipment and high sensitivity. Chen et al. developed a simple and sensitive electrochemical biosensor for detection of the DNA species related to ORAOV1[12]. But until now, nearly all DNA based electrochemical biosensors need

tedious electrode modification, where the signal probe must be immobilized on the electrode surface, these procedures are time consuming; also cause effect to the reproducibility of the biosensor. And the DNA hybridization occurred on the solution-electrode interface, this heterogeneous strategy suffers from low efficiency because of the spatial hindrance effect of the electrode surface. Thus, it is very necessary to develop a homogeneous immobilization free system for DNA detection.

DNA nicking endonuclease Nt.BstNBI can recognize a simple asymmetric sequence (5'-GAGTC-3'), and cleave only one DNA strand at the 4-bases away from the 3' end of its recognition site. Since nicking endonuclease requires complete complementarity between DNA strand and the target at the restriction site and sufficient complementarity to allow hybridization outside of the enzyme recognition site, so it owns high specificity and can be used to detect single-base mismatch and single nucleotide polymorphisms. Kiesling et al had reported a novel nucleic acid detection technique called nicking endonuclease signal amplification (NESA) based on this principle[13], the identification ability to target DNA of NESA is much better than that of the exonuclease III due to the highly specificity of nicking reaction. Li et al had showed a NESA based fluorescent biosensor included basic version and extended version for DNA dection, the detection limit of the tow versions respectively are 6.3 pM and 85 fM[14]. Many other DNA biosensors had been developed based on NESA[15,16]. All these biosensors showed the high amplification efficiency and high spectivity of NESA.

Early report showed that a negatively charged ITO surface can be achieved after

particular treating processes[17]. Due to the negatively charged phosphate on DNA molecular skeleton, DNA also contains negative charge[18]. Short DNA contains little negative charge while long DNA contains big negative charge, so short DNA can diffuse freely to the negatively charged ITO surface while the long DNA cannot reach the ITO surface easily because of the difference of the electrostatic repulsion. This character had been applied to develop immobilization free biosensors for different targets[19-21]. And which had been coupled with the amplification procedures to achieve sensitive target detection. For examples, I-Ming Hsing et al developed an immobilization-free electrochemical sensor for DNA detection combined with exonuclease III enhanced signal amplification tactics[22]. However, the selectivity of these sensors are not high enough, especially its distinction ability to single-base mismatch or double-bases mismatch. Furthermore, the sensitivities are also not high enough, even when exonuclease III enhanced signal amplification tactics was used.

To the best of our knowledge, no report which combines the advantages of high identification ability of NESA and ITO based homogeneous immobilization free electrochemical biosensor had been reported. In this study, we demonstrate a homogeneous immobilization free, ultra-high sensitive and selective electrochemical biosensor for the detection of target DNA species related to ORAOV1 in saliva, which combined the convenient the immobilization free of ITO based biosensor and high specific and amplification efficiency of NESA. The established biosensor has high specificity and can be used to indentify a single-base mismatch and single nucleotide polymorphisms (SNPs).

EXPERIMENTAL SECTION

Reagents. All oligonucleotides were synthesized by Sangon Inc. (Shanghai, China). Their sequences are shown below:

methylene blue (MB) labeled substrate strand (eMB): 5'-AAAGAACGAGAGTCTTTC ↓ TG-MB-3' (the underlined letters are the recognition sequence of Nt.BstNBI and the arrow indicates the nicking position);

target DNA (T1): 5'-CAGAAAGACTCTCGTTCTTT-3';

single-base mismatched DNA (T2): 5'-CAGAAAGACT**G**TCGTTCTTT-3';

double-base mismatched DNA (T3): 5'-CAGAAAGAC**GG**TCGTTCTTT-3';

non-complementary DNA (T4): 5'-GCTCCCTTCAGAGCAATCCC-3';

(T5):5'-AAAAACAGAAAGACTCTCGTTCTTTAAAAA-3'(include T1);

(T6):5'-AAAAAAAAAACAGAAAGACTCTCGTTCTTTAAAAAAAAAA-3'(include T1).

(T7):5'-AAAAAAAAAAAAAAACAGAAAGACTCTCGTTCTTTAAAAAAAAAAAAAAA-3'(include T1)

T5, T6 and T7 all contain the same DNA sequence of target DNA and have extra various numbers of bases at each end of the target DNA sequence. The nicking enzyme (Nt.BstNBI) and NEBuffer 3 (50 mM Tris-HCl, 10 mM MgCl2, 100 mM NaCl, 1 mM dithiothreitol, pH 7.9) were obtained from New England BioLabs, and used directly without further purification. All other chemicals were of analytical grade.

Electrochemical detection system. Electrochemical measurements were performed

with a computer controlled CHI660a electrochemical system (CH Instruments, Shanghai, China) in a conventional three electrode electrochemical cell with an ITO as the working electrode, Pt as the reference electrode and counter electrode. The potential of the Pt reference electrode in the buffer was determined to be +0.36 V with respect to an Ag/AgCl reference electrode. Before electrochemical detection, the ITO electrode was sonicated in an alconox solution (10g/L of alconox of double-distilled water) for 15 min, propan-2-ol for 15 min, and twice in double-distilled water for 15 min in sequence. After these treating processes, a negatively charged working electrode surface can be accomplished.[23]

NESA based DNA detection. A volume of 50 μL of 1×NEBuffer 3 containing 1.0 μM eMB probe, 10 units of Nt.BstNBI, and different concentrations of the target DNA, 1.0 nM of the single-base-mismatched DNA, 1.0 nM of the double-base-mismatched DNA, or 1.0 nM noncomplementary DNA was incubated at 37 ℃ for 1 h. Then the above mentioned solution had been incubated at 55 ℃ for a certain period of time. Then, the differential pulse voltammetry (DPV) signal from the mixture was recorded. The DPV that had a potential interval of 0 to -0.6 V was carried out in buffer 3. Each sample had been detected for five times and the average value had been applied for quantitative analysis.

Saliva collection and artificial saliva sample. Saliva, a complicated biological fluid, contains interfering material such as minerals, electrolytes, DNA, RNA and proteins, had been chosen as an example. Unstimulated whole saliva samples were collected between nine and eleven in the morning.[24] The subjects were asked to keep on an

empty stomach and refrain from oral hygiene procedures for at least 1 hour before the collection. Saliva samples were centrifuged at 2,600 rpm for 10 minutes at 4 °C with TGL-16 Table-type High-speed Refrigerated Centrifuge (Hunan instrument co., LTD). 1 mL the supernatant had been taken out and diluted for 10 times with 1×NEBuffer 3. Then blended the artificial saliva sample by mixing the dilute supernatant with different concentrations of target DNA T1.

RESULTS AND DICUSSIONS

Principle of the homogeneous immobilization free electrochemical biosensor. The principle of proposed homogeneous immobilization free NESA based electrochemical DNA biosensor is shown in Fig.1. The approach realize immobilization free by taking advantage of the differential diffusivity between electroactive reporter-tagged long DNA and short DNA toward a negatively charged ITO electrode surface and the high specificity of NESA. The designed eMB consists of a 20-mer DNA strand and a methylene blue tag labeled at the 3' terminus. The eMB has the simple asymmetric sequence which can be recognized by nicking endonuclease. In the absence of the target DNA, the eMB can't be recognized by nicking endonuclease and nicking reaction can't proceed. And the eMB, due to the strong electrostatic repulsion from the negative ITO electrode, negligible electrochemical response can be detected on the ITO working electrode. In the presence of target DNA, the eMB hybridizes with target DNA to form double-stranded structure, which can be recognized by nicking endonuclease. So which can be cleaved into two parts, one part contains 18-base(LD)

and the other one contains only 2-base. The 2-base ssDNA had been linked with methylene blue(eT), since the electrostatic repulsion from the negatively charged ITO working electrode to eT released is much littler than that from eMB, so a remarkable enhanced electrochemical response can be detected. At the same time, target DNA can dissociate from the ds-DNA and trigger the next round of hybridization, cleavage and releasing. In this way, a single target DNA can repeatly trigger the strand-scission cycle for many times and realize the signal amplification.

A simple assistant experiment has been performed to verify our presumption. As shown in Fig.2, in the absence of the target DNA, the signal from methylene blue was negligible (curve a). That is to say, the DPV signal from the eMB is little due to its abundant negative charged. In the presence of target DNA, a remarkable enhanced electrochemical response was observed (curve b). The reasons lie in that little negative charged eTs have been released after interaction with target DNA, which can diffuse easily to the negative charged ITO electrode surface, which results in the enhancement DPV signal detected. Thus, the aforementioned results clearly demonstrated the feasibility of our strategy for DNA assay.

Optimization of the reaction conditions. The initial concentration of eMB plays an important role in the performance of the biosensor. Excessive amount of eMB can lead to high background signal, and lower concentration will cause the very low detection signal, which affects the sensitivity of the proposed sensor. So the effect of eMB concentration had been optimized firstly. The concentration of the target DNA was set at 10 pM and the concentrations of eMBs were changed from 0.2 to 2 μM, the

results showed that the DPV signal of the system increased with the substrate concentration and then reached the saturated condition after 0.8 μM (see Fig.3(A)). And if the concentration of substrate was too high, the background signal will grow. Therefore, 1.0 μM eMB has been chosen as the optimized condition.

The effects of the reaction time and the incubation temperature of nicking reaction on the signal detected were investigated too. As shown in Fig.3(B), the DPV signal increased with the increasing of reaction time and reached a plateau after 30 min. As anticipated, nicking reaction was extremely rapid. Therefore, 30 min had been chosen as the optimized condition for the following study. Fig.3(C) showed that the DPV signal increased with an increment of incubation temperature up to 55 °C. However, the DPV signal decreased if the temperature changed to 70 °C. The reason lies in that LD and target hardly melt and the enzyme's activity was low when the incubation temperature below 55 °C, which below the unwinding condition (T_m = 66.8 °C) and the optimal temperature of the enzyme (T = 55 °C). And the enzyme will become inactive if the incubation temperature enhanced further, which results in the decreasing of signal detected. So, 55 °C was selected as a compromise.

Calibration curve and reproducibility of the biosensor. The DPV responses from the system contains different concentration of target DNA were detected to evaluate the sensitivity and dynamic range of the as-prepared electrochemical biosensor. As shown in Fig.4(A), the DPV signal increased gradually with the increasing of target DNA concentration. This phenomenon is in accord with the fact that with higher target DNA concentration, more eMBs were cleaved, resulting in the increasing of the

faradic current. Moreover, the DPV signal displayed a good linear relationship with target DNA concentration in the range from 1 pM to 10 pM (see Fig.4(B)). The regression equation is:

$$\Delta I\ /\mu A = -1.4812*10^{-2} - 5.4014*10^{-3} Cx/pM,\ R = 0.9977$$

Where ΔI (the difference of the currents detected at present and absent of target) is enhancement of the DPV current, Cx is target concentration, and R is the regression coefficient. The limit of detection was estimated to be 0.35 pM (S/N=3). Compared with the existing immobilization free electrochemical DNA biosensors based on the exonuclease III-amplification[25], the proposed DNA biosensor showed higher sensitivity due to high efficiency of the nicking endonuclease signal amplification and signal on mechanism.

Interference Study. The selectivity of the proposed sensor had been investigated by using eMB to hybridize with various DNA sequences (target DNA, single-base-mismatch DNA T2, double-base-mismatch DNA T3, and non-complementary DNA T4 mentioned in reagents section). As shown in Fig.5(A), the electrochemical signal is negligible despite of the addition of interfering reagent at a concentration of 1.0 nM. However, it was found that the proposed biosensor had an obvious response with respect to 8 pM target DNA. And the ΔI produced by 8 pM target DNA is about 7 folds than that from 1.0 nM T2, which confirmed the proposed biosensor owns better selectivity than others homogeneous DNA electrochemical biosensor reported (I-Ming Hsing et al, 1 fold; Liu et al, 2 folds) [20,22]. The results clearly indicated that the biosensor had high distinction ability to single-base

mismatch, double-base mismatch and noncomplementary DNA sequences. The excellent selectivity of the method was due to the high specificity of nicking endonuclease to special asymmetric DNA sequence and target recycling. Additional, we also used eMB to hybridize with another DNA sequences (T5, T6, T7) to study the specificity of the biosensor further. As shown in Fig.5(B), the electrochemical signal has no obvious changing between target DNA, T5, T6 and T7. It demonstrated that extra various amounts of bases at the each end of the unbroken target DNA sequence have no effect on specificity of the developed biosensor. These indicated the developed immobilization free electrochemical DNA biosensor here could be applied for the ultrasensitive detection of target DNA from saliva sample with high specificity. Moreover, this strategy can be used to detect a single-base mismatch and single nucleotide polymorphisms (SNPs).

Application of the biosensor in artificial saliva sample. The proposed sensor has been applied to detect target DNA species added in artificial saliva sample. As shown in Table 1, the recoveries were in the range of 99.4 - 101.7%, the relative standard deviations (RSDs) were 5.7 - 6.4%. These results indicate that the proposed biosensor can be applied to test the target in complex samples with satisfied results.

CONCLUSION

In summary, we developed a simple, rapid, immobilization-free and ultra-high selective electrochemical biosensor to determine target DNA species. It takes advantage of the signal amplification of nicking endonuclease assisted target

recycling and the differential diffusivity between electroactive reporter-tagged long DNA and short DNA toward a negatively charged ITO electrode surface. Unlike most existing electrochemical DNA sensors, our designed sensor don't need the tedious probe immobilization processes. What's more, due to the high spectivity of nicking endonuclease assisted target recycling, the proposed biosensor exhibits high discrimination ability even against a single-base mismatch. Furthermore, since DNA hybridization and recognization were all performed in the homogeneous solution, which owns the character of high efficiency. The proposed biosensor can be applied to the quantitative determination of DNA and expected to detect single nucleotide polymorphisms (SNPs). The proposed method can be applied to detect other DNA sequences which contain the recognition site of the Nt.BstNBI, such as the DNA from Bacillus subtilis. Furthermore, with the selection of corresponding specific nicking endonucleases and carefully designed probe sequences, the proposed method can be extended to detect other specific DNA sequences.

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Figures and Captions

Fig.1 Mechanism of the proposed homogeneous immobilization free electrochemical biosensor for DNA detection based on NESA

Fig.2 DPV responses of reaction mixtures containing 1μM eMB, 0.2 unit/μL Nt.BstNBI, and 10 pM target DNA (curve b) or 0 pM target DNA (curve a) in 1×NEBuffer 3, after incubation at 37°C for 1h and then warm to 55 °C for 30min.

Fig.3 (A) Effect of the eMB concentration on the detection system. (B) The effect of the reaction time of nicking reaction on the DPV signal detected. (C) The effect of incubation temperature of nicking endonuclease on the DPV signal detected. [eMB] = 1μM, [Nt.BstNBI] = 0.2 unit/μL, [target DNA] = 10 pM.

Fig.4 (A) DPV responses of reaction mixtures containing 1μM eMB probe, 0.2 unit/μL Nt.BstNBI and varying concentrations of target DNA after incubation at 37 °C for 1 h and then warm to 55 °C for 30 min. (B) The current increment plotted against concentration of target DNA. From a to f: 0 pM, 1 pM, 4 pM, 6 pM, 8 pM, 10 pM.

Fig.5 (A) Specificity of the electrochemical biosensor: (a) 8 pM target, (b) 1.0 nM single-base mismatched DNA, (c) 1.0 nM double-bases mismatched DNA, (d) 1.0 nM noncomplementary DNA, and (e) no target. [eMB] = 1μM, [Nt.BstNBI] = 0.2 unit/μL. (B) Specificity of the electrochemical biosensor: (a) 8 pM target, (b) 8 pM T5, (c) 8 pM T6, (d) 8 pM T7. [eMB] = 1μM, [Nt.BstNBI] = 0.2 unit/μL.

Figure 1

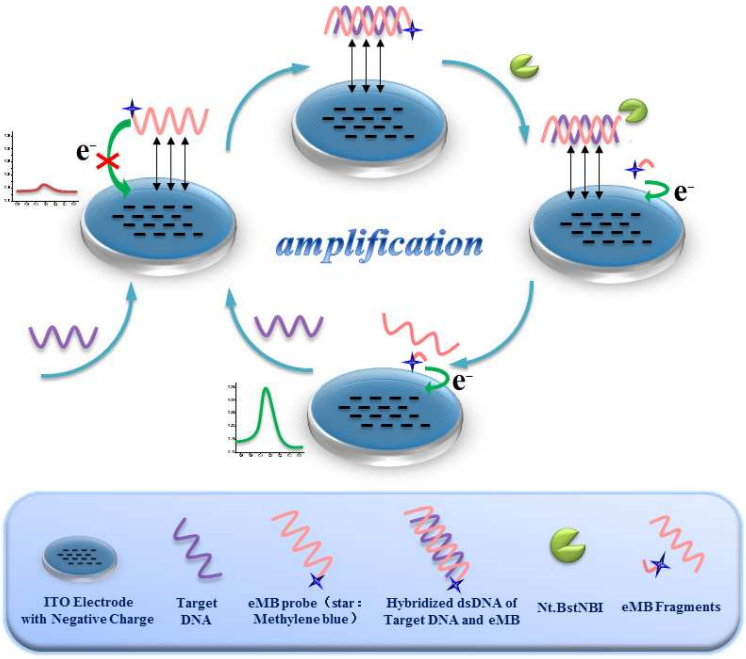
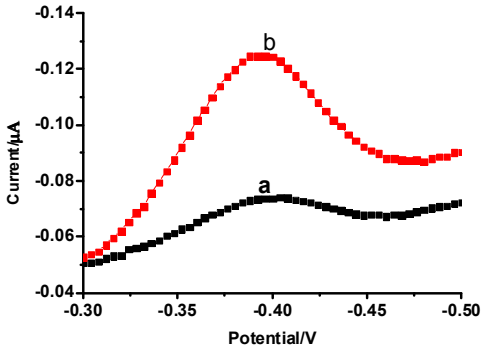

e⁻
amplification
e⁻
e⁻
ITO Electrode with Negative Charge
Target DNA
eMB probe（star：Methylene blue）
Hybridized dsDNA of Target DNA and eMB
Nt.BstNBI
eMB Fragments

Figure 2


-0.14
-0.12
-0.10
-0.08
-0.06
-0.04
Current/μA
-0.30
-0.35
-0.40
-0.45
-0.50
Potential/V
a
b

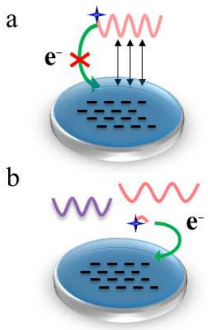

a
e⁻
b
e⁻

Figure 3

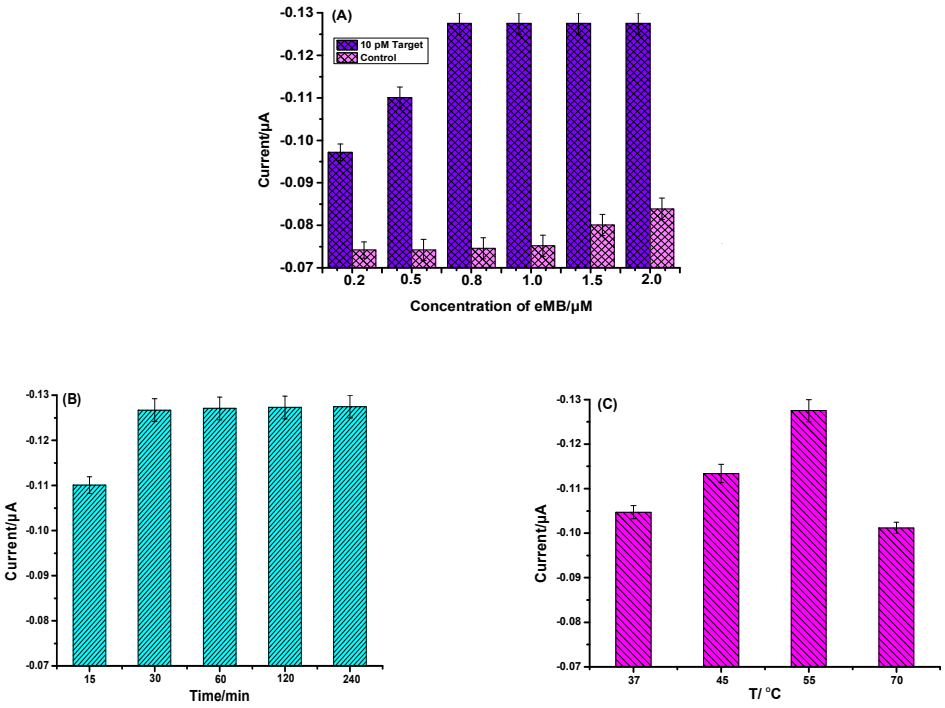

(A)
10 pM Target
Control
Current/μA
-0.13
-0.12
-0.11
-0.10
-0.09
-0.08
-0.07
0.2
0.5
0.8
1.0
1.5
2.0
Concentration of eMB/μM
(B)
Current/μA
15
30
60
120
240
Time/min
(C)
Current/μA
37
45
55
70
T/ °C

Figure 4

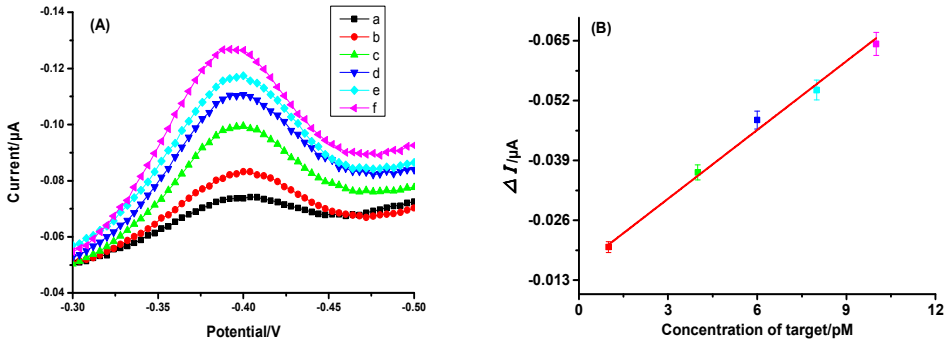

(A)
a
b
c
d
e
f
Current/μA
Potential/V
-0.14
-0.12
-0.10
-0.08
-0.06
-0.04
-0.30
-0.35
-0.40
-0.45
-0.50
(B)
Δ I /μA
Concentration of target/pM
-0.065
-0.052
-0.039
-0.026
-0.013
0
3
6
9
12

Figure 5

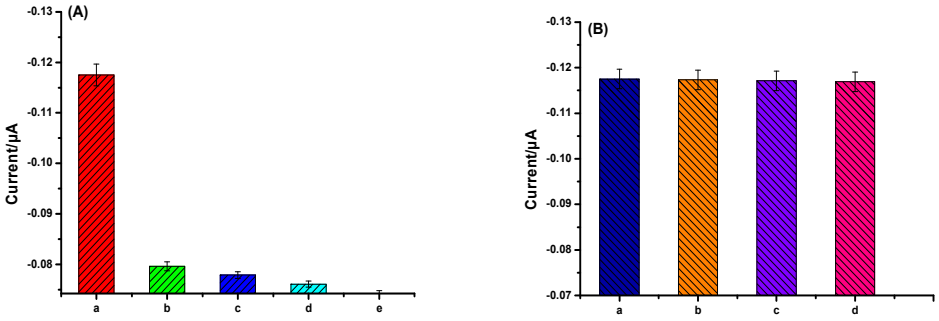
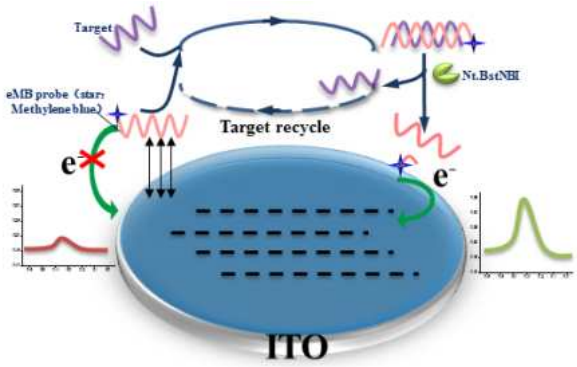

(A)
Current/μA
-0.13
-0.12
-0.11
-0.10
-0.09
-0.08
a
b
c
d
e
(B)
Current/μA
-0.13
-0.12
-0.11
-0.10
-0.09
-0.08
-0.07
a
b
c
d

Table 1 Detection of target DNA added in artificial saliva samples (n=5)

Sample No.	Target DNA added(pM)	Target DNA detected (pM)	Recovery (%)	RSD (%)
1	3.00	3.05	102	6.3
2	5.00	4.97	99.4	5.7
3	8.00	8.11	101	6.1

For TOC Only


Target
Nt.BstNBI
eMB probe (start
Methylene blue)
Target recycle
e
e
ITO