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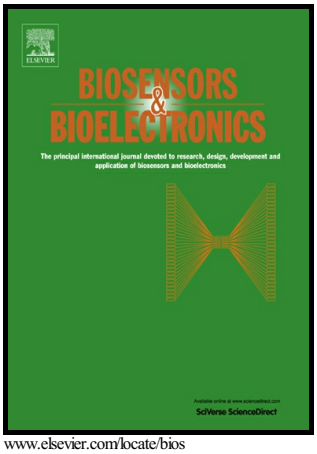

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Electrochemical DNA biosensor based on grafting-to mode of terminal deoxynucleoside transferase-mediated extension

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Electrochemical DNA biosensor based on grafting-to mode of terminal deoxynucleoside transferase-mediated extension

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Abstract

Previously reported electrochemical DNA biosensors based on in-situ polymerization approach reveal that terminal deoxynucleoside transferase (TdTase) has good amplifying performance and promising application in the design of electrochemical DNA biosensor. However, this method, in which the background is significantly affected by the amount of TdTase, suffers from being easy to produce false positive result and poor stability. Herein, we firstly present a novel electrochemical DNA biosensor based on grafting-to mode of TdTase-mediated extension, in which DNA targets are polymerized in homogeneous solution and then hybridized with DNA probes on BSA-based DNA carrier platform. It is surprising to find that the

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background in the grafting-to mode of TdTase-based electrochemical DNA biosensor have little interference from the employed TdTase. Most importantly, the proposed electrochemical DNA biosensor shows greatly improved detection performance over the in-situ polymerization approach-based electrochemical DNA biosensor.

Keywords: electrochemical DNA biosensor, terminal deoxynucleoside transferase, background, in-situ, grafting-to

1 Introduction

DNA is an important biomarker for early diagnosis and prognosis of genetic diseases, including cancer. Recently, DNA assay has attracted increasing attention and many efforts have been devoted to DNA biosensors (Giljohann and Mirkin, 2009; Newman et al., 2014; Sassolas et al., 2008). However, DNA biomarkers are presented at trace level in human bodies, which is a barrier for further applications of most proposed methods in real samples. Hence, it is urgent to develop ultrasensitive biosensors to detect DNA biomarkers in very low abundance. Polymerase chain reaction (PCR) (Kiss et al., 2008), the most popular technique for amplifying nucleic acids, has been widely employed in various fields. However, the need for specific equipment, relatively long assay time, and high cost largely limit its application in resource-limited settings and for point-of-care (POC) analysis. Isothermal amplification techniques (Ali et al., 2014; Dean et al., 2001; Deiman et al., 2002; Guo et al., 2009; Höfer et al., 2013; Jia et al., 2010; Murakami et al., 2012; Ness et al., 2003; Nimitphak et al., 2000; Paez et al., 2004; Shi et al., 2014; Tomita et al., 2008;

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Vincent et al., 2004) without thermocycling requirement have emerged as a promising alternative to PCR for rapid and efficient amplification of nucleic acid. However, these methods suffer from needing multiple enzymes and complex design. It is worth noting that terminal deoxynucleoside transferase (TdTase) (Chow and Chilkoti, 2007) can directly catalyze the incorporation of multiple dNTP or small molecule conjugated dNTP (e.g., Cy3-dNTP and biotin-dNTP) into a single-strand DNA (ssDNA) with 3'-OH terminal under isothermal condition, hence augment the signal turnover with its advantage of extremely simple preparation process, flexible design and versatile application.

Among different types of DNA biosensors (optics, electrochemistry, surface plasmon resonance, quartz crystal microbalance, and surface acoustic microbalance) (Liu et al., 2015; Pei et al., 2012; Wang et al., 2013; Xuan et al., 2015; Zagorodko et al., 2014), the electrochemical DNA sensor has a unique appeal as a promising tool for point-of-care diagnostics due to its remarkable features of simplicity, portability and inexpensive instrumentation, high sensitivity and selectivity, fast response as well as low fabrication cost. Up to now, some electrochemical DNA sensors using TdTase amplification technique have been reported (Hu et al., 2015; Mei et al., 2016; Shen et al., 2015; Wan et al., 2013, 2014; Yang et al., 2014; Yuan et al., 2014). Su et al (Wan et al., 2013) have had the first report of electrochemical DNA (E-DNA) sensor based on in-situ polymerization, also known as surface initiated enzymatic polymerization (SIEP), by employing TdTase, and the results indicated a good amplifying performance of this simple E-SIEP DNA sensor. Unfortunately, a high background

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occurred in this E-SIEP DNA sensor, and although a unique measure of treating the electrode with KOH was taken, the results were not satisfied. Also, in Tjong's (Tjong et al., 2011) study of amplified on-chip fluorescence detection of DNA hybridization by SIEP, they found that the nonspecific signal from immobilized probes also increased with the increasing ratio of fluorescent dNTP to its corresponding natural dNTP when exposing the probe to the TdTase reaction mixture containing fluorescent dNTP in the absence of a bound target, for reasons that they do not yet understand. Therefore, in order to get a lower detection limit, it is better to clearly figure out the origin of the high background in the E-SIEP DNA sensor and find a more proper way to couple TdTase with electrochemical DNA sensor.

In this work, we delicately investigate the origin of high background in E-SIEP DNA sensor, and find it is compelled to reduce the amount of TdTase for obtaining low background. Interestingly, when we made first attempt to label the target with dNTPs prior to hybridization in E-DNA sensor, which we named grafting-to mode vividly, we find the background value would not increase with the increasing amount of TdTase. Most importantly, this E-DNA sensor based on grafting-to mode of TdTase-mediated extension, compared with the E-SIEP DNA sensor, can not only maintain low background, but also significantly enhance the detection performance.

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2 Experimental

2.1 Reagents and materials

Bovine serum albumin (BSA) was obtained from Shanghai Sangon Biological

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Engineering Technology and Services Co., Ltd. (China). Terminal deoxynucleoside tansferase (TdTase), 10X reaction buffer (500 mM KAc, 200 mM $Mg(Ac)_2$, 100 mM Tris-HAc, pH 7.9) and 10X (2.5 mM) solution of $CoCl_2$ were purchased from New England Biolabs. Ltd. (Beijing, China). Biotinylated 2'-dexyuridine 5'-triphosphate (biotin-dUTP) and 2'-dexyadenosine 5'-triphosphate (dATP) were from Thermo Scientific (Shanghai, China). Avidin horseradish peroxidase (Av-HRP) was from Roche Diagnostics (Mannheim, Germany). Neogen Enhanced K-blue substrate was obtained from Neogen (US).

The synthetic oligonucleotides were purchased from TaKaRa Inc. (Dalian, China) with base sequences as follows:

Probe: 3'-SH-$(CH_2)_6$-T_{10}CAAGGCTGATGACGGAGTGGGTAT-5'

Target: 3'-GCTATACCCACTCCGTCATCAGCCTTG-5'

The synthetic oligonucleotides were dissolved in TE buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 M NaCl to desired stock concentrations and stored at -20 ^{o}C. The buffer solutions employed in this study were as follows: probe DNA immobilization buffer was also the TE buffer mentioned above. The washing buffer was 10 mM PB buffer (pH 7.4). Av-HRP was diluted by using 10 mM PBS buffer (pH 7.4, 0.1 M NaCl) containing 0.25 % BSA. The chemicals were of analytical grade and used as received without further purification. All aqueous solutions were prepared and diluted using ultrapure water (18.2 MΩ/cm) obtained from Millipore Milli-Q system.

2.2 Preparation of electrochemical biosensor

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The ssDNA-BSA-based biosensor was fabricated following the process of literature (Liu et al., 2013). Briefly, the gold electrode was immersed in 0.1 % BSA for 15 min at room temperature (25 ^{o}C) after polish and electrochemical cleaning. Then, a 3 μL volume of 1 μM capture probe was dropped onto the BSA modified gold electrode for 1 h at room temperature. Finally, the sensor was thoroughly rinsed with PB buffer and dried with nitrogen for further use.

2.3 In-situ polymerization approach

The ssDNA-BSA-modified electrode was incubated with hybridization buffer containing target DNA for 1 h at 45 ^{o}C before extension by TdTase. After that, the electrode was immersed into a total volume of 50 μL mixed solution (5.0 μL reaction buffer, 5.0 μL $CoCl_2$, 0.25 μL biotin-dUTP, 2.5 μL dATP, 0.1 μL TdTase, 37.15 μL ddH_2O) and performed for 2 h at 37 ^{o}C. After being washed, a 3 μL volume of Av-HRP (0.5 U/mL) was spread on the electrode surface for 15 min at room temperature (25 ^{o}C). Finally, the electrode was extensively rinsed and subjected to electrochemical measurements.

2.4 Grafting-to mode approach

Unlike in-situ polymerization approach, in this part the TdTase-mediated extension reaction and signal addition was performed in a homogeneous solution for 20 minutes at 37 ^{o}C (50 μL volume of mixed solution containing 5.0 μL reaction buffer, 5.0 μL $CoCl_2$, 0.25 μL biotin-dUTP, 0.75 μL dATP, 0.5 μL TdTase, 5.0 μL target DNA, and 33.5 μL ddH_2O, respectively). After that, the ssDNA-BSA-modified electrode was dipped into aforementioned solution for 1 h at 50 ^{o}C. After being

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washed, a 3 μL volume of Av-HRP (0.5 U/mL) was spread on the electrode surface for 15 min at room temperature (25 ^{o}C). Finally, the electrode was extensively rinsed and subjected to electrochemical measurements.

2.5 Electrochemical measurements

An electrochemical analyzer (CHI760D, Chenhua Co., Shanghai, China) was used for amperometric experiments, and a conventional three-electrode configuration was employed all through the experiments, which involved the modified AuE as the working electrode (2 mm diameter), a platinum wire as the auxiliary electrode and an Ag/AgCl as the reference electrode. By using current-time technique, the voltage was fixed at 100 mV and the electroreduction current was measured at 100 s in Neogen Enhanced K-blue substrate.

3 Results and discussions

3.1 Working principle of the two TdTase-based electrochemical DNA sensors

In present work, two different E-DNA sensors employing TdTase were depicted (Scheme 1). The first type was the typical one that adopted the in-situ polymerization approach, while the other one used the grafting-to mode proposed firstly to construct E-DNA sensor. At beginning, both of the sensors were self-assembled with BSA molecules, and then capture probes labeled with thiols at their 3’ terminals were immobilized on the gold surface in the interval of BSA molecules via Au-S chemistry. Subsequently, in the former sensor, TdTase was used to catalyze the sequential addition of dNTPs at the 3’-OH group of target DNAs after hybridization, while in the

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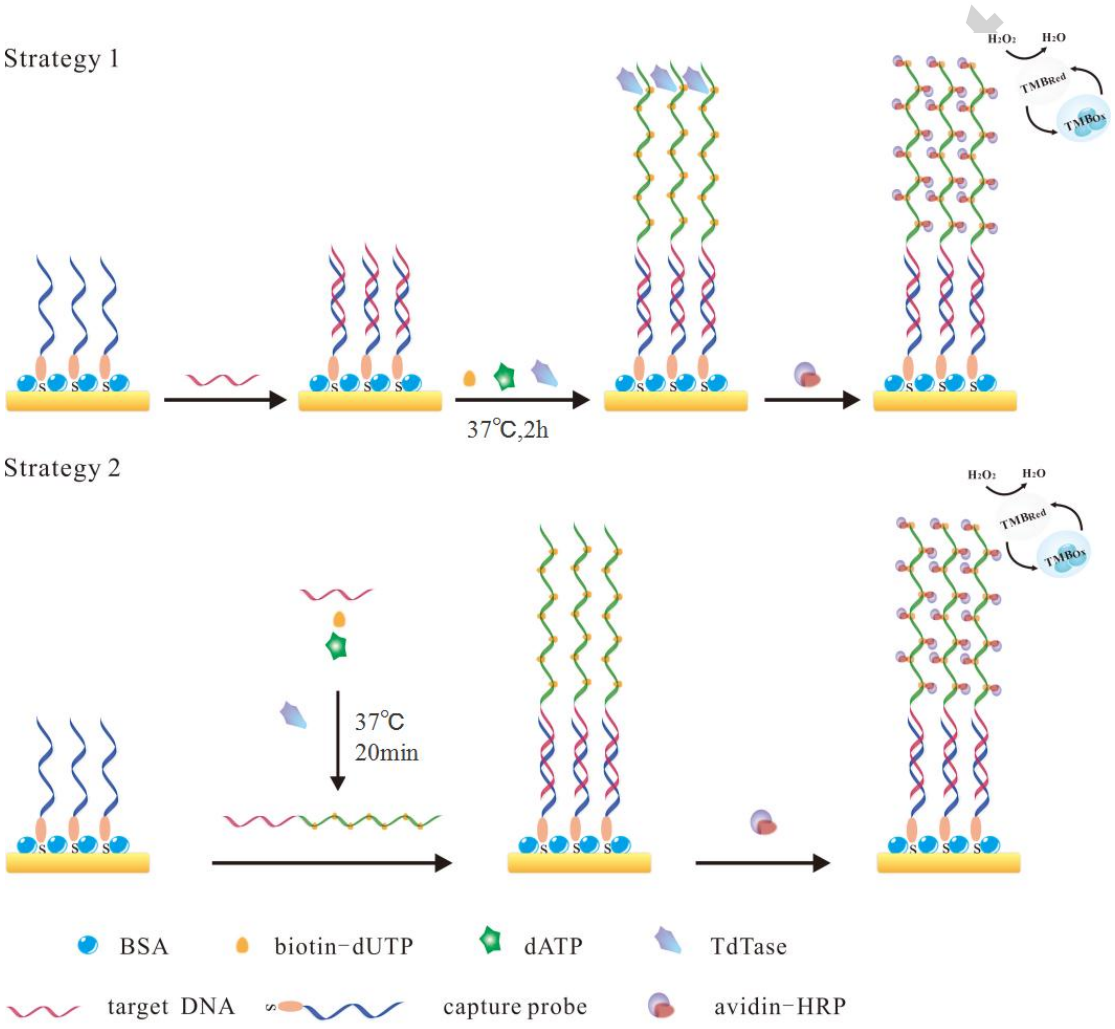
latter sensor, TdTase-catalyzed DNA polymerization was carried out in solution, and then the extended products were hybridized with the capture probes on the gold electrode surface. By using a mixture containing dATP and biotin-dUTP, biotin labels were incorporated into the generated long target ssDNAs. Av-HRPs were then specifically bound to the biotin labels via biotin-avidin bridge. Finally, we interrogate the electric current generated by HRP catalyzed reduction of hydrogen peroxide contained in the TMB substrate for quantitatively readout of the target DNA.



Scheme 1. Schematic illustration of the working principle of two different modes of TdTase-based electrochemical DNA sensors.

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3.2 Comparison of background between the in-situ polymerization approach and the grafting-to mode approach

In the E-DNA sensor based on the in-situ polymerization approach of TdTase-mediated extension, Su et al (Wan et al., 2013) supposed that the high background value was caused by the nonspecific adsorption of capture probes. In their opinion, failure to fully labeling 3'-terminal of DNA with thiols provided nonspecific target for TdTase, thus it led to nonspecific extension. Although a treatment using KOH to remove nonspecific adsorption was adopted, the results were not satisfied. Also, means by applying potential and competitive method were tried to eliminate nonspecific adsorption of DNA probes, but they didn't work in reducing background value.

Peptide nucleic acid (PNA), an oligonucleotide (ON) mimic, has nucleobases attached to a simple, non-natural polyamide backbone that consists of alternating ethylene diamine and glycine units. Therefore, PNA, due to without 3'-OH groups, can be an ideal substitution for DNA capture probes in the E-SIEP sensor since it is not a substrate of TdTase. However, results shown in Figure 1 indicate that the background of PNA-based E-SIEP sensor was still high, and so was the same as that of the BSA modified electrode. These results show that the action of TdTase on the gold electrode rather than the nonspecific adsorption of DNA probes gave rise to the background. In order to further verify this speculation, the effect of varying the amount of TdTase on the background was examined. As shown in Figure 2a, in the E-DNA sensor based on the in-situ polymerization approach of TdTase-mediated

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extension, when the unit of TdTase diminished in the reaction mixture, the amperometric current significantly decreased and reached its minimal level until 2 unit of TdTase was employed. In contrast, background value in the E-DNA sensor based on the grafting-to mode of TdTase-mediated extension always kept low as the unit of TdTase varied from 2 to 10 (Figure 2b), indicating this developed method, where the background had little interference from the amount of employed TdTase, featured robustness over the E-SIEP DNA sensor.

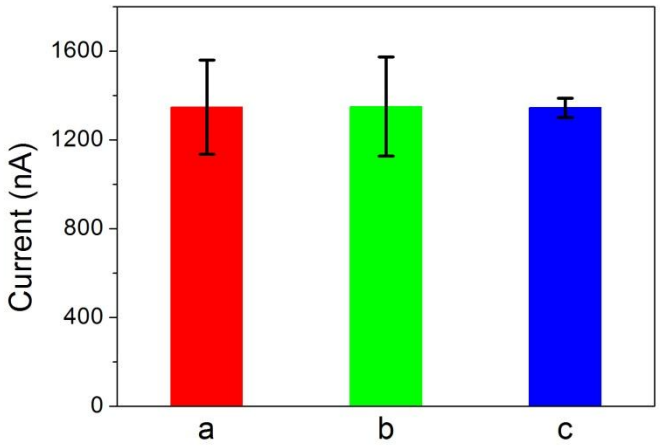

Figure 1. Current responses of TdTase/ssDNA/BSA/AuE (a), TdTase/ssPNA/BSA/AuE (b), TdTase/BSA/AuE (c), respectively.

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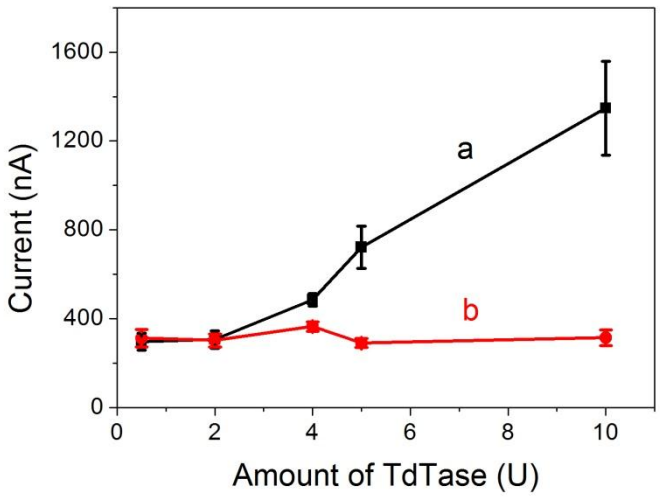

Figure 2. Effect of the amount of TdTase on the background in the in-situ polymerization approach (a) and the grafting-to mode approach (b).

3.3 Optimization of the ratio of biotin-dUTP to dATP

The synthetic DNA fragments extended from 3' terminal of target DNA contain continuous biotin-dUTPs, however, the degree of closeness between biotin-dUTPs will affect the binding efficiency of Av-HRP to them owing to steric hindrance. To the best of our knowledge, there is little information available in literature about the investigations on the binding of Av-HRP to a string of ordered biotin, thus the ratio of biotin-dNTP to dNTP was firstly examined in this study. According to the previous report (Chow and Chilkoti, 2007), the nucleotide preference of TdTase in SIEP was dATP > dTTP > dGTP ≈ dCTP, thus dATP was selected for condition optimization. In the case of grafting-to mode approach (Figure 3), response from biotin-dUTP/dATP ratio of 0/1 in the presence of 1 nM target DNA had no difference with that in the absence of target DNA, suggesting that the specific binding between biotin-dUTP and

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Av-HRP resulted in subsequent reaction leading to current generation. In addition, the binding efficiency was limited when biotin-dUTP was used as the sole monomer in the mixture. As seen in Figure 3, the highest signal-to-noise ratio, up to 30 times, was achieved at 1/3 of biotin-dUTP/dATP, indicating that dATPs were used as embedded molecules so that the distance between biotin-dUTPs was favorable for binding of Av-HRP to biotin-dUTP. These results significantly showed signal amplification could work when a certain amount of dATPs acted as inserting agent benefiting for Av-HRP's greatly effective binding to biotin-dUTP along with the synthetic DNA fragments.

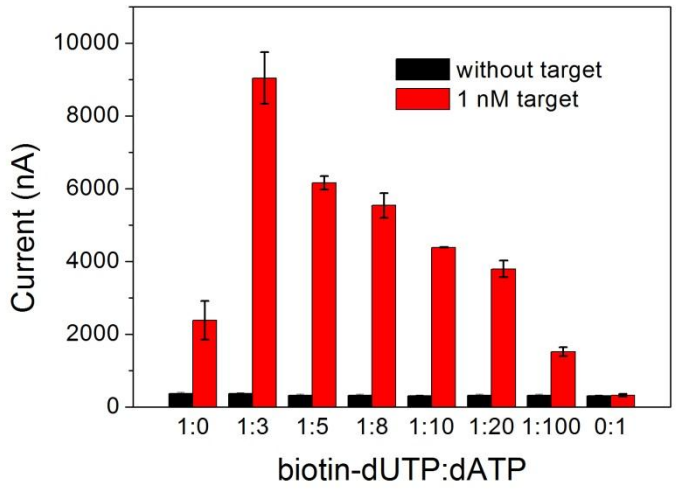

Figure 3. Optimization of the ratio of biotin-dUTP/dATP in the grafting-to mode approach.

3.4 Kinetic investigation in grafting-to mode approach

As shown in Figure 2b, the background in grafting-to mode approach was unaffected by the increasing amount of TdTase, and given that the amount of TdTase

would be the rate-limiting factor of this enzyme reaction under these conditions, we carried out kinetic investigation in the presence of different amount of TdTase. As demonstrated in Figure 4, in the case of 2 U TdTase, the current intensity increased slowly with the extension time and reached a plateau until 120 minutes, while the case of 10 U TdTase showed a dramatic increase and completed the extension rapidly in 20 minutes due to the elevated unit of TdTase. Thus, these observations indicated the extension mediated by TdTase could be finished in a short time by employing higher TdTase in homogeneous solution polymerization on the premise of low background in this developed E-DNA biosensor.

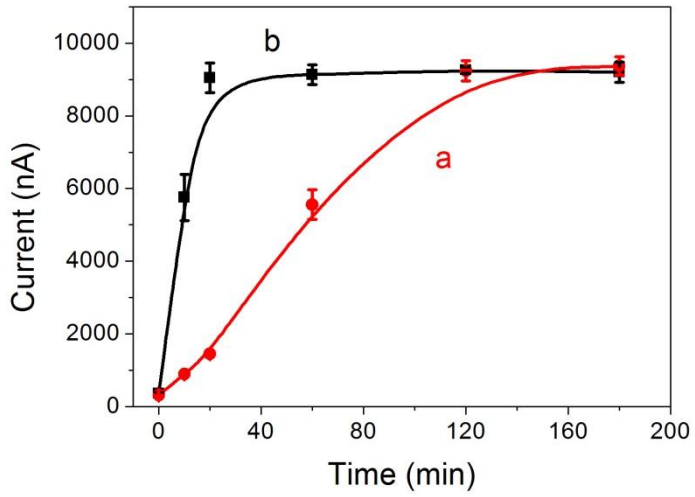

Figure 4. Plots of current as a function of extension time by using 2 U (a) or 10 U (b) TdTase in the presence of 1 nM target DNA.

3.5 Comparison of detection performance between the in-situ polymerization approach and the grafting-to mode approach

The method of in-situ polymerization synthesizes DNA fragments after

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hybridization with DNA probes on surface, where only the hybridized target DNAs are polymerized so that it enables the in-situ synthesis of dense polymer brushes at the surface, but as mentioned above, the background in this method is significantly affected by the amount of TdTase, which is confronted with being easy to produce false positive result and poor stability. Differently, we firstly attempt to synthesize DNA fragments in homogeneous solution and then employ these extended DNAs to hybridize with DNA probes on surface, where it is a mode of grafting-to. Although this grafting-to mode approach may result in a low density of polymer chains at the surface, due to steric and thermodynamic constraints, results (Figure 2b) indicate the background in this approach had little interference from the employed TdTase. Afterwards, under the optimized experimental conditions, the detection performance of these two TdTase-based E-DNA sensors was compared from aspects of background, time cost, and signal intensity. As depicted in Figure 5, although a high response was obtained in 20 minutes in the in-situ polymerization approach by using 10 U TdTase, the background value was very high, and measures of reducing amount of TdTase must be taken, leading to a low response and time-consuming. Therefore, the employment of the grafting-to mode in E-DNA biosensor had remarkable advantages of low background, short time, high sensitivity. For this merit, we tentatively believe that it was ascribed to a well established BSA-based DNA probe carrier platform (Liu et al., 2013) for facile controlling the inter-probe and probe-target interactions on a surface so that the extended DNAs were grafted from solution to surface with high efficiency. Besides, the polymerization efficiency in homogeneous solution must be

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better than that in heterogeneous system. Thus, these two factors made the detection performance of the grafting-to mode approach significantly better than that of the in-situ polymerization approach in E-DNA biosensor.

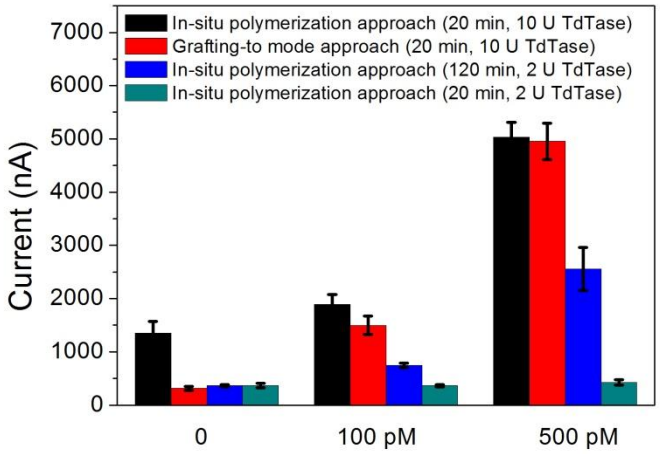

Figure 5. Comparision of detection performance between the in-situ polymerization approach and the grafting-to mode approach in electrochemical DNA biosensor.

4 Conclusions

In summary, a novel electrochemical DNA biosensor based on grafting-to mode of TdTase-mediated extension was constructed. Different from the previously reported DNA electrochemical biosensor based on the in-situ polymerization approach by employing TdTase, also known as SIEP, the proposed method introduced multiple signaling molecules to achieve amplification through the way of homogeneous extension prior to hybridization on the electrode surface. The proposed sensor could effectively avoid the generation of high background and complete the overall reaction in 20 min. The comparisons in the aspects among background, time cost and signal

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intensity showed the proposed method had significantly improved detection performance over the in-situ polymerization approach-based electrochemical DNA biosensor.

Acknowledgments

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Highlights

- Electrochemical DNA biosensor based on grafting-to mode of TdTase-mediated extension.
- The origin of high background in E-SIEP DNA sensor was delicately analyzed.

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- Effect of steric hindrance on binding efficiency between avidin-HRP and biotin-dUTP.
- The ratio of biotin-dUTP to dATP was firstly examined in this study.
- Significantly improved detection performance over in-situ polymerization approach.

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