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Exonuclease-Catalyzed Methylene Blue Releasing and Enriching onto Dodecanethiol Monolayer for Immobilization-Free and Highly Sensitive Electrochemical Nucleic Acid Biosensor

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Abstract

Herein, a unique and versatile immobilization-free electrochemical nucleic acid biosensor architecture is proposed for the first time based on the catalyzed releasing of methylene blue (MB)-tagged mononucleotide by exonuclease III (Exo III) and the successive enriching onto dodecanethiol monolayer, which could be attributed to the hydrophobic force between the alkyl chain of dodecanethiol monolayer and the hydrophobic part of MB-tagged mononucleotide. The fabricated biosensor demonstrates the considerable advantages including assay simplicity, rapidness and high sensitivity owing to its immobilization-free and homogenous operation for the biorecognition and amplification process. A low detection limit of about 1 pM toward target DNA could be achieved with an excellent selectivity. The proposed immobilization-free electrochemical biosensing strategy was also extended for the assay of exonuclease I and III activity. Furthermore, it might be easily extended for the detection of a wide spectrum of targets, and thus provide a promising avenue for the development of immobilization-free and sensitive electrochemical biosensors.

Keywords: Immobilization-free; Electrochemical biosensor; Nucleic acid; Target recycling; dodecanethiol monolayer

Introduction

The electrochemical biosensor has been received substantial efforts and shown promising applications in molecular recognition, medical diagnosis and environmental monitoring owing to its prominent features including simplicity, low cost, high sensitivity, ease of calibration and compatibility with miniaturization technologies.[1-4] As to the typical electrochemical biosensor fabrication, the immobilization of bio-recognition element onto transducer surface constitutes a primary and essential step.[5-7] It accordingly imposes the challenge for the screening of suitable immobilization method, especially for the method that could effectively regulate the assembly density of bio-recognition element. The delicate control of immobilization conditions or post-modification by small molecules are often adopted for the optimal biosensing performance.[8-11] However, such relatively complex immobilization or optimization procedures are not beneficial for the reproducibility and stability of the fabricated biosensor. Also, the bio-recognition and signal amplification would be easily influenced by the electrode surface, which definitely limits the detection performance toward analytes. In this context, the advance of immobilization-free electrochemical biosensor is highly pursued.

Currently, several immobilization-free electrochemical biosensing mechanisms have been advocated.[12-15] For example, Wu et al. developed an electrochemical molecular beacon for homogenous detection of target DNA.[16] The carminic acid was labeled at both ends of molecular beacon and it could self-associate into dimers for the quenched electrochemical signal. The opening of molecualr beacon by target DNA made the conversion from electrochemically inactive dimer to active monomer for the target response. However, the limited electroactive aromatic compounds that could self-associate into dimers would restrict the wide application of this method. Another important contribution for immobilization-free electrochemical biosensing was proposed by Hsing

group,[17] which was based on the diffusion difference between electro-active species labeled DNA strand and its digestion product by exonuclease (electro-active species labeled mononucleotide) toward the negative charged electrode surface. Till now, this immobilization-free sensing strategy has been widely employed for the electrochemical detection toward different targets.[18-20] However, the diffusion difference for the detection mechanism might be more easily influenced by the environmental factors for example pH, ionic strength, and especially by relatively complex biological matrix. Also, the lack of inherent connection between signal reporter and electrode surface might be not beneficial for the detection reliability and reproducibility toward analytes. Another immobilization-free electrochemical nucleic acid biosensing strategy was developed by Zhuang et al. based on the isothermal strand displacement amplification-activated anti-streptavidin aptamer,[21] which could be captured by the immobilized streptavidin on the electrode surface. Although no DNA immobilization step was involved, the streptavidin immobilization on the electrode surface was necessary. It thus might exert the influence on the recognition efficiency between anti-streptavidin aptamer and streptavidin, and further on the detection performance toward nucleic acid owing to the steric hindrance. Therefore, the development of new immobilization-free electrochemical biosensing strategy is still highly desirable.

Herein, inspired by the previous studies that the methylene blue (MB) molecule could be effectively trapped at the self-assembly monolayer (SAMs) of dodecanethiol by hydrophobic force between the alkyl chain of dodecanethiol and the hydrophobic part of MB molecules,[22-24] a novel and versatile immobilization-free electrochemical biosensing strategy was proposed based on the exonuclease III (Exo III)-catalyzed MB-labeled mononucleotide releasing and the successive enriching onto dodecanethiol monolayer. Exo III is a

sequence-independent enzyme, which can catalyze the stepwise removal of mononucleotides from 3′-terminus of double-stranded DNA in the case of substrate with a blunt or recessed 3′-terminus.[25-28] It shows limited activity toward single-stranded DNA or duplex DNA with a protruding 3′-terminus. Thus, compared with endonuclease-based sensing method, exonucleases, especially Exo III could provide a more versatile platform for amplified DNA detection.[29,30] In current sensing system, a 3′-MB labeled hairpin probe (HP) was designed to possess a protruding ssDNA fragment at the 3′-terminus, which could resist the digestion by Exo III. In the presence of target DNA, its recognition with the MB-labeled HP triggered the Exo III cleavage process, accompanied with the target recycling and the releasing of MB-labeled mononucleotide. The MB-labeled mononucleotide could be trapped into dodecanethiol monolayer by hydrophobic interaction between the alkyl chain of dodecanethiol and the hydrophobic part of MB-tagged mononucleotide, whereas it could not work for the intact MB-tagged HP owing to its big size and the hydrophilic skeleton of HP DNA, which could prevent the interaction of terminal-attached MB with alkyl chain of dodecanethiol monolayer. On the basis of Exo III-catalyzed MB-tagged mononucleotide releasing and its enriching onto dodecanethiol monolayer, the nucleic acid recognition process could be carried out in the solution rather than on the electrode surface, which would not only accelerate DNA interaction in the enzymatic reaction but also improve the biorecognition efficiency. Furthermore, current immobilization-free electrochemical strategy could be extended for the assay of exonuclease I (Exo I) and exonuclease III (Exo III) activity. It thus would hold a huge potential for the development of immobilization-free and sensitive electrochemical biosensors.

Experimental Section

Materials and Chemicals. The dodecanethiol were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Exonuclease III (Exo III),

Exonuclease I (Exo I) and 10×NEBuffer 1 (1×NEBuffer 1, 10 mM bis-tris-propane-HCl, 10 mM $MgCl_2$, 1mM dithiothreitol, pH 7.0 at 25 °C) were purchased from New England Biolabs, Inc. (Ipswich, MA, USA). Fetal calf serum was obtained from Sangon Biotech. Co., Ltd. (Shanghai, China). Acrylamide/bisacrylamide 39:1 40% gel stock solution, N,N,N′,N′tetramethylethylenediamine (TEMED), and ammonium persulfate (APS) were purchased from Yantai Science and Biotechnology Co., Ltd. (Yantai, China). T safe™ dye were purchased from Shanghai Tianneng Science and Technology Co., (Shanghai, China). The HPLC-purified 3′-methylene blue (MB)-modified DNA and other oligonucleotide sequences were purchased from Sangon Biotech. Co., Ltd. (Shanghai, China) and listed in Table S1. All other reagents were of analytical grade without further purification.

Gold Electrode Treatment and Self-Assembly of Dodecanethiol. The gold electrode was firstly polished with 0.05 μm alumina powder on a microcloth to obtain a mirror surface, followed by sonication in ethanol and water for 5 min, respectively. Then, it was electrochemically cleaned in a 0.5 M H_2SO_4 solution within a potential window between -0.2 and +1.5 V at a scan rate of 100 mV s^{-1}. The self-assembly of dodecanethiol monolayer was performed by immersing the above cleaned gold electrode into 1 mM dodecanethiol ethanolic solution for 14 h at room temperature.

Exonuclease III (Exo III)-Catalyzed Digestion Process for Target Detection. For the detection of target DNA, 50 μL 1×NEBuffer 1 containing 1 μM MB-labeled HP, 50 units of Exo III, and varying concentrations of target DNA was incubated at 37 °C for 45 min and then heated at 70 °C for 15 min to deactivate the Exo III. After that, the dodecanethiol monolayer assembled electrode was incubated into the above solution for 10 min to enable the adsorption of the released MB-tagged mononucleotide onto the electrode surface. Then, the electrochemical measurements were recorded in 10 mM PBS solution

(pH 7.4, 0.1 M NaCl). For the assay of Exo III activity, 1 μM MB-labeled HP was firstly hybridized with 1 μM target DNA in 50 μL 1×NEBuffer 1 for 60 min at 37 °C. Then, varying units of Exo III was added for another 30 min for the assay of Exo III activity.

Exonuclease I (Exo I) Activity Assay. 50 μL 1×NEBuffer 1 containing 1 μM MB-labeled ssDNA and varying units of Exo I was incubated at 37 °C for 30 min. Then, the dodecanethiol monolayer assembled electrode was incubated into the above solution for 10 min to enable the adsorption of the released MB-tagged monucleotide onto the electrode surface.

Electrochemical Characterizations. All electrochemical experiments were performed with a conventional three-electrode system comprising a gold working electrode, a platinum wire auxiliary electrode, and a Ag/AgCl reference electrode. After MB enriching onto the dodecanethiol monolayer assembled electrode, the cyclic voltammetry (CV) and differential pulse voltammetry (DPV) experiments were performed in 10 mM PBS solution (pH 7.4, 0.1 M NaCl). The CV curves were scanned from -0.5 V to 0.2 V with a scan rate of 50 mV/s. The DPV curves were scanned from 0 V to -0.5 V with the pulse amplitude and period of 50 mV and 0.2 s, respectively. The EIS experiments were measured in 10 mM $Fe(CN)_6^{3-/4-}$ solution containing 1 M KCl with the frequency range from 0.1 Hz to 10 kHz. Before electrochemical measurements, the electrolyte solution should be thoroughly purged with high purity nitrogen for about 20 min to avoid the interference from the reduction of oxygen.

Polyacrylamide Gel Electrophoresis. To prepare the hydrogel, 3.5 mL 40% gel solution (39:1), 160 μL 50×TAE Buffer, 80 μL 10% APS, 4 μl TEMED and 4256 μL deionized water were mixed. This mixture contained a final gel percentage of 17.5%. The polyacrylamide gel electrophoresis (PAGE) was carried out in 1×TAE buffer at 180 V for 3

min and then 135V for 90 min, followed by staining in T safe™ dye solution.

Apparatus. The electrochemical experiments were performed with a CHI 660D electrochemical workstation (CH Instruments, Shanghai, China). The gels were scanned using the FR-980A gel image analysis system (Shanghai, China).

Results and Discussion

Detection Principle for the Immobilization-Free Strategy. It had been previously verified that the methylene blue (MB) molecule could be effectively captured by the self-assembled monolayer (SAM) of 1-dodecanethiol based on the hydrophobic interaction between the alkyl chain of 1-dodecanethiol and the hydrophobic part of MB molecule.[22-24] Also, considered with the wide applications of MB as the electro-active label, especially in the electrochemical nucleic acid biosensor fabrication, herein, a novel immobilization-free electrochemical biosensing strategy is proposed on the basis of Exo III-catalyzed MB releasing and its enriching onto the dodecanethiol monolayer, which is schematically illustrated in Figure 1. The critical step underlying this approach takes advantage of the association difference between MB-tagged mononucleotide and oligonucleotide onto the dodecanethiol monolayer. A 3′-MB labeled hairpin-like DNA probe (HP) is designed, which contains a protruding ssDNA fragment at 3′-terminus and can effectively resist the specific cleavage by Exo III. The target DNA can hybridize with the HP to form a partially double-stranded DNA structure with a blunt 3′-terminus. Then, the Exo III can bind to the duplex region and catalyze the stepwise removal of mononucleotides from the 3′-terminus, liberating the MB-tagged mononucleotides. The released MB-tagged mononucleotide could then be captured onto the dodecanethiol monolayer by the hydrophobic force between the alkyl chain of dodecanethiol and the hydrophobic part of MB-tagged mononucleotide for the electrochemical response toward target DNA. On the other hand, the target DNA is intact due to its protruding 3′ terminus and will be released accompanied with the

digestion of the MB-tagged HP probe. The released target DNA is then able to be "recycled" and hybridize with a new uncleaved HP to initiate a new cycle of HP digestion. In this way, a single target DNA is able to trigger the digestion of multiple HP probes into the accumulated MB-tagged mononucleotides for the generation of a remarkable electrochemical signal toward target DNA. In the absence of target DNA, the MB-tagged mononucleotide could not be efficiently liberated and the intact MB-tagged HP would not be effectively adsorbed onto the dodecanethiol monolayer owing to its big volume and the hydrophilic skeleton of oligonucleotide, which could prevent the interaction of terminal-attached MB with alkyl chain of dodecanethiol monolayer on the electrode surface. Although the assembly step of dodecanethiol monolayer on the electrode surface is involved, it should be noted that such self-assembly process for the formation of dense and well-organized monolayer could be easily operated. Also, the bio-recognition and Exo III-catalyzed cleavage reaction is completely conducted in the solution phase. The assembled dodecanethiol monolayer ingeniously serves as a predator toward the released MB-tagged mononucleotide. Thus, the immobilization-free and highly sensitive electrochemical analysis toward targets could be readily achieved by current Exo III-catalyzed MB releasing and enriching effect.

<<Herein for Figure 1>>

Detection Feasibility of Immobilization-Free Electrochemical Nucleic Acid Biosensor. The feasibility of developed immobilization-free electrochemical nucleic acid biosensor for target DNA detection was firstly verified and shown in Figure 2. The Figure 2A shows the corresponding cyclic voltammetric (CV) responses in the presence and absence of 100 nM target DNA. A well-defined redox peak of MB with the anodic and cathodic potential located at -186 mV and -250 mV, respectively, was obtained in the presence of target DNA. A close inspection of the redox peaks of MB versus different scan rates revealed that the peak current increased in linear correlation to the scan rate, featuring a typical surface-controlled

electrochemical process. This also confirmed that MB had been captured onto the dodecanethiol monolayer assembled electrode (Figure S1). However, in the case of no target DNA, only very weak redox peak could be observed, indicating that Exo III-catalyzed releasing of MB-tagged mononucleotide and the successive adsorption onto dodecanethiol monolayer was largely inhibited. The DPV responses were in well accordance with the results of CV (Figure 2B). The reduction peak current of MB in the presence of 100 nM target DNA was about 5.2 fold of that at no target DNA. The electrochemical responses obtained at different control experimental conditions were also shown in Figure 2C. In the presence of only HP or the coexistence of HP and target DNA, the MB-tagged HP could not efficiently adsorb onto the dodecanethiol monolayer owing to its big volume and hydrophilic skeleton of oligonucleotide, which restricted the interaction of terminal-attached MB with alkyl chain of dodecanethiol monolayer. With the further introduction of Exo III but no target DNA, the electrochemical response was increased compared with that in the absence of Exo III, which could be attributed to the non-specific cleavage of Exo III toward single-stranded DNA for the releasing of some MB-tagged mononucleotides.[31] However, it is far below the electrochemical response obtained in the presence of Exo III and target DNA.

To further verify that the electrochemical signal was indeed due to the adsorbed MB-tagged mononucleotide onto the dodecanethiol monolayer, the comparative experiments were also conducted by directly using MB as the substitute of MB-tagged mononucleotide. It could be found from Figure S2 that, after incubation of dodecanethiol monolayer assembled electrode into 1 μM MB solution, the well-defined redox peaks of MB could be also obtained, indicating again that the MB could be effectively trapped onto the dodecanethiol monolayer by hydrophobic force. Interestingly, the electrochemical response after adsorption of MB-tagged mononucleotide was more evident than that by adsorption of MB (Figure S2B). The detailed mechanisms were not still clear for now.

The Exo III-catalyzed target recycling process was also verified by gel

electrophoresis images (Figure 2D). The Lanes 1 and 2 showed the respective bands for the HP and target DNA. A bright band with an obviously decreased migration shift was observed for the hybrids of HP with target DNA (Lane 3). In the presence of Exo III but no target DNA, the band for the HP could be evidently imaged, indicating that no distinct cleavage toward HP by Exo III occurred (Lane 4). In the case of target DNA and Exo III, only the band for target DNA could be observed, but the bands for the HP and the hybrids of HP with target DNA disappeared together (Lane 5), verified that the HP had been completely digested by Exo III in the presence of target DNA.

<<Herein for Figure 2>>

The immobilization-free biosensor fabrication on the dodecanethiol monolayer assembled electrode was further confirmed by electrochemical impedance spectroscopy (EIS) and CV characterizations (Figure 3). The EIS data were fitted by an equivalent electrical circuit (inset, Figure 3A). Only a very small charge transfer resistance (Rct) of 301.1 Ω could be observed for the bare gold electrode (curve a in Figure 3A), indicating a very rapid charge transfer process. Also a pair of quasi-reversible redox peaks of $Fe(CN)_6^{3-/4-}$ could be obtained (curve a in Figure 3B). After the assembly of dodecanethiol monolayer on the electrode surface, a very big semicircle with the Rct value of 30.88 KΩ was obtained (curve b in Figure 3A). The diffusion peaks of $Fe(CN)_6^{3-/4-}$ were also completely inhibited (curve b in Figure 3B). It could be attributed that the well-organized and densely packed monolayer of dodecanethiol largely blocked the diffusion and electron transfer of $Fe(CN)_6^{3-/4-}$ toward electrode surface. Furthermore, with the trapping of MB-tagged mononucleotides into the dodecanethiol monolayer after Exo III-catalyzed target recycling process, the Rct value was decreased to be about 12.81 KΩ (curve c in Figure 3B), indicating that the electron transfer of $Fe(CN)_6^{3-/4-}$ toward electrode surface was dramatically improved. This could be explained that the intercalated MB molecule into dodecanethiol monolayer played the electron tunnelling role to promote the electron transfer of $Fe(CN)_6^{3-/4-}$ toward electrode surface. Also, the redox current of $Fe(CN)_6^{3-/4-}$

after MB intercalation (curve c in Figure 3B) was obviously larger than that on the pure dodecanethiol monolayer modified electrode (curve b in Figure 3B). The EIS and CV experiments further provided beneficial evidence for the trapping of MB-tagged mononucleotide onto dodecanethiol monolayer after Exo III-catalyzed digestion process, which constituted the immobilization-free electrochemical biosensing mechanism.

<<Herein for Figure 3>>

Optimization of Experimental Conditions. In order to reveal the best sensing performance, the experimental conditions including the MB-labeled HP concentration, reaction temperature and reaction time were optimized. Firstly, the employed MB-labeled HP concentration would have an important effect on the electrochemical response toward target DNA. Three MB-labeled HP concentrations were employed to simply explore the electrochemical response toward target. The electrochemical signal toward target DNA increased with the increase of employed MB-labeled HP concentration. The background signal also increased accordingly with the increase of HP concentration. The HP concentration of 1 μM could achieve a larger signal to background ratio (S/B) toward 10 nM target DNA than that at other two concentrations (Figure 4A). Thus, 1 μM of HP was advised for current immobilization-free electrochemical detection of targets. The reaction temperature for the Exo III-catalyzed digestion process was also investigated (Figure S3). The electrochemical response at 37 ^{o}C was better than that at 25 and 45 ^{o}C, which could be easily explained that the Exo III could work more efficiently at 37 ^{o}C. The time response for the Exo III-catalyzed digestion process was also electrochemically monitored (Figure 4B). The background signal was only slightly increased with the studied incubation time from 0 to 90 min. The electrochemical intensity increased evidently with time and almost reached the saturation value at 45 min when using a 10 nM target DNA. The continuously increased signal indicated that the Exo III-catalyzed target recycling process indeed took place and the signal saturation at 45 min suggested that the MB-labeled HP probe was completely consumed.

<<Herein for Figure 4>>

Detection Performance of Immobilization-Free Electrochemical DNA Biosensor. Under the optimized experimental conditions, the detection performance of the fabricated immobilization-free electrochemical DNA sensor was investigated by using a series of different concentrations of target DNA ranged from 0 to 10 nM. As shown in Figure 5A, the dynamically increased DPV peak current with increasing target DNA concentration indicated that the Exo III-catalyzed releasing of MB-tagged mononucleotide and the successive trapping into the dodecanethiol monolayer was highly dependent on the concentration of target DNA. A good linear relationship between the electrochemical intensity and the logarithm value of the target DNA concentration ranged from 1 pM to 10 nM could be obtained (Figure 5B). The regression equation was Y = 110.7 + 8.29 lgX (Y and X represented the DPV peak current and target DNA concentration, respectively; unit of X was M) with the correlation coefficient of 0.9974. The detection limit, which was defined as 3 times the standard deviation of the background value, was about 1 pM. It was lower than that of some developed immobilization-free electrochemical DNA biosensors (Table S2). Also, the homogenous bio-recognition and the successive enriching of MB for current immobilization-free biosensor could be separately implemented, which would endow the target detection with a robust reliability and reproducibility. Whereas most immobilization-free electrochemical biosensing strategies are directly operated in the sample system,[16,17] which would be more easily influenced by the environmental factors. Furthermore, a relatively big volume of sample or a specially fabricated microelectrode is often required for these developed immobilization-free strategies. Although the detection limit for current immobilization-free method was still higher than that of most heterogenous DNA biosensors, it demonstrated considerable advantages particularly in terms of assay simplicity and rapidness. The relative standard deviations (RSDs) toward three different target DNA concentrations of 1 pM, 100 pM and 10 nM were obtained as 7.3%, 8.4% and 5.7%, respectively, based

on five repetitive measurements, indicating a good reproducibility of our proposed immobilization-free strategy for target DNA detection. The detection specificity of the developed immobilization-free electrochemical DNA biosensor was further investigated by using four kinds of DNA sequences, including complementary target DNA (TD), single-base mismatched DNA (1MT), three-base mismatched DNA (3MT), and non-complementary DNA (NC), at the same concentration. As shown in Figure 5C, the electrochemical responses of the biosensor toward 1MT and 3MT after substracting the background signal were about 56% and 33% of that for perfect target DNA. The NC showed almost the same value with that of the blank solution. Thus, the proposed immobilization-free electrochemical biosensing strategy exhibited a good performance to discriminate the perfect complementary target from the base-mismatched targets. The target DNA detection was also performed when the ascorbic acid (AA), uric acid (UA) or dopamine (DA) was coexisted in the tested sample (Figure S4). These electroactive species showed no evident interference toward the target DNA detection. To further verify the detection feasibility of the fabricated biosensor in the relatively complex sample matrix, the detection of target DNA was also conducted in the 5% diluted fetal bovine serum (Figure 5D). It could be seen that the electrochemical responses in the diluted serum was generally lower than that in the buffer. It might be caused by the weak adsorption of some serum matrixes onto the monolayer, which restricted the adsorption of released MB-tagged mononucleotide to some extent. However, the electrochemical intensity also increased continually with the increase of the spiked target DNA concentration. The target DNA with the concentration as low as 5 pM could be readily detected in the diluted serum, indicating the potentiality of the developed sensing system for DNA detection in relatively complex biological samples. Furthermore, the whole biosensing system is only involved into a one-step self-assembly of dodecanethiol monolayer, which would endow the robust stability compared with that of some biomolecules immobilized electrode. Our experiments showed that the

dodecanethiol monolayer assembled electrode possessed almost the same electrochemical response toward target DNA-triggered MB-tagged mononucleotide releasing and enriching events even after its storage in the refrigerator for over one month.

<<Herein for Figure 5>>

Detection Versatility of Fabricated Immobilization-Free Electrochemical Biosensor. Actually, any targets that could induce the releasing of MB-tagged mononucleotide could be detected by currently proposed immobilization-free strategy. The immobilization-free electrochemical method was also empolyed for the assay of Exo III activity. The DNA duplex with the blunt 3′-MB terminus was firstly prepared by hybridizing MB-labeled HP with target DNA, which would be cleaved to release MB-tagged mononucleotides only in the presence of Exo III. Thus, the Exo III activity could be electrochemically followed after enriching of MB-tagged mononucleotides onto dodecanethiol monolayer. The detection performance toward Exo III activity was shown in Figure S5. The electrochemical responses increased continually with increasing Exo III concentration from 0 to 1000 U mL^{-1}. An experimental detection limit of about 2 U mL^{-1} could be obtained. Furthermore, the exonuclease I (Exo I) was used as another model for its activity assay by currently proposed immobilization-free electrochemical method. Exo I can catalyze the digestion of single-stranded DNA from 3′ to 5′ and plays an important function in the maintenance of genomic stability.[32,33] The detection principle for Exo I activity is schematically illustrated in Figure 6A. A single stranded DNA with the MB label at 3′-terminus is employed as signal carrier. In the absence of Exo I, such MB-labeled ssDNA could not adsorb onto the dodecanethiol monolayer assemblied electrode for the electrochemical response. The relatively big volume and more hydrophilic characteristics of whole skeleton of MB-labeled ssDNA would not be beneficial for the hydrophobic interaction between dodecanethiol monolayer and terminal-attached MB of ssDNA. In the presence of Exo I, the MB-labeled ssDNA could be stepwise digested from 3′ to 5′ to release the MB-tagged

mononucleotide, which would be well trapped into the dodecanethiol monolayer by hydrophobic interaction for the electrochemical response toward Exo I. The assay feasibility for Exo I activity was shown in Figure 6B. A distinct electrochemical response of MB could be obtained at 1000 U mL^{-1} Exo I and only very weak electrochemical signal of MB could be observed in the absence of Exo I. The detection performance for the Exo I activity was also investigated by using different concentrations of Exo I. The electrochemical intensity increased continually with the increase of Exo I concentration from 0 to 1000 U mL^{-1} (Figure 6C). An experimental detection limit of 1 U mL^{-1} could be readily achieved. Above this concentration, the DPV peak current showed a good linear relationship with the Exo I concentration from 1 to 30 U mL^{-1}. The detection limit toward exonuclease activity is relatively higher than that of previously reported fluorescence methods (Table S3). However, current immobilization-free electrochemical method provides a promising avenue for the simple and rapid assay of exonuclease activity.

<<Herein for Figure 6>>

Conclusions

In conclusion, a novel immobilization-free and sensitive electrochemical nucleic acid sensing strategy was developed based on the Exo III-catalyzed releasing of MB-tagged mononucleotide and successive enriching onto dodecanethiol monolayer assembled electrode. It takes fully advantages of the difference of hydrophobic interaction between MB-tagged mononucleotide and oligonucleotide with alkyl chain of dodecanethiol monolayer. It features considerable advantages over traditional electrochemical biosensors, particularly in terms of assay simplicity, rapidness and high sensitivity owing to the homogenous operation for target recognition and amplification. A low detection limit of about 1 pM toward target DNA could be achieved with an excellent selectivity. Furthermore, it was extended for the assay of Exo I and Exo III activity. It thus would hold a huge potential for the development of immobilization-free and sensitive electrochemical biosensors.

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Notes

The authors declare no competing financial interest.

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ASSOCIATED CONTENT

Supporting Information. Additional information including the DNA sequences used in the experiment (Table S1), the effect of scan rate on the cyclic voltammetric response (Figure S1), the electrochemical responses for the methylene blue onto dodecanethiol monolayer (Figure S2), the effect of reaction temperature on electrochemical response (Figure S3), the interference experiment (Figure S4), the Exo III activity assay (Figure S5) and the detection performance comparison with reported methods (Table S2 and 3). This information is available free of charge via the Internet at http://pubs.acs.org/

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Figure Captions

Figure 1. Schematic illustration of electrochemical nucleic acid biosensor fabrication based on Exo III-catalyzed target recycling followed with the MB releasing and enriching effect.

Figure 2. The CV (A) and DPV (B) responses for the fabricated immobilization-free biosensor toward blank and 100 nM target DNA in 10 mM PBS (pH 7.4, 0.1 M NaCl), respectively. The CV curves were scanned from -0.5 V to 0.2 V with the scan rate of 50 mV/s. The DPV curves were scanned from 0 V to -0.5 V with the pulse amplitude and period of 50 mV and 0.2 s, respectively. (C) The bar chart for the DPV responses obtained at different control conditions. The employed concentrations for HP and Exo-III were 1 μM and 1U/μL, respectively. (D) Non-denaturing PAGE analysis: lane 1, 10 μM HP; Lane 2, 10 μM target DNA; Lane 3, 4 μM HP and 4 μM target DNA; Lane 4, 4 μM HP and 4 U/μL Exo-III; Lane 5, 4 μM HP, 4 μM target DNA and 4 U/μL Exo-III.

Figure 3. EIS (A) and CV (B) responses recorded in 10 mM PBS (pH 7.4, 1 M KCl) and 10 mM $Fe(CN)_6^{3-/4-}$ solution for the different modified electrodes. The curves were obtained for bare gold electrode (a), dodecanethiol monolayer assembled electrode (b), and after trapping of MB-tagged mononucleotide (c). The concentration of target DNA was 100 nM. The impedance plots were obtained with the frequency range from 0.1 Hz to 10 KHz. The scan rates for the CV were 100 mV/s. The inset in the Figure 3A showed the corresponding equivalent electrical circuit. The magnified plots for the curves b and c in Figure 3B were also shown in the inset.

Figure 4. (A) The optimization of employed HP concentration toward the detection of 10 nM target DNA. Three different HP concentrations including 0.5, 1.0 and 2.0 μM were studied. (B) The time dependency of the electrochemical responses toward blank and 10 nM target DNA.

Figure 5. (A) DPV responses corresponding to the analysis of different concentrations of target DNA. The employed target DNA concentration: a, 0 M; b, 1 pM; c, 5 pM; d, 20 pM; e, 100 pM; f, 500 pM; g, 2 nM; h, 10 nM, respectively. (B) The linear relationship between the DPV peak current and the logarithm value of the target DNA concentration. Error bars represented standard deviations of measurements (n = 3). (C) Selectivity tests of the

fabricated DNA biosensor toward 100 nM of complementary target DNA (TD), single-base mismatched DNA (1MT), three-bases mismatched DNA (3MT), noncomplementary DNA (NC), and the absence of DNA (Blank). Error bars represented standard deviations of measurements (n = 3). The inset showed the corresponding DPV responses obtained at different DNAs. (D) DPV responses obtained at different concentrations of target DNA spiked in 5% diluted fetal calf serum and 1×NEBuffer 1.

Figure 6. (A) Schematic illustration for the Exo I activity assay based on the Exo I-catalyzed releasing of MB-tagged mononucleotide and its successive enriching effect on the dodecanethiol monolayer. (B) The DPV responses recorded in 10 mM PBS (pH 7.4, 0.1 M NaCl) for the sensing system in the presence and absence of Exo I. The MB-tagged single-stranded DNA and Exo I concentrations were 1 μM and 1000 U mL^{-1}, respectively. (C) The calibration curve between DPV responses and Exo I concentration ranged from 1 U mL^{-1} to 1000 U mL^{-1}. The inset showed the linear relationship between the DPV peak current and the Exo I concentration. Error bars represented standard deviations of measurements (n = 3)

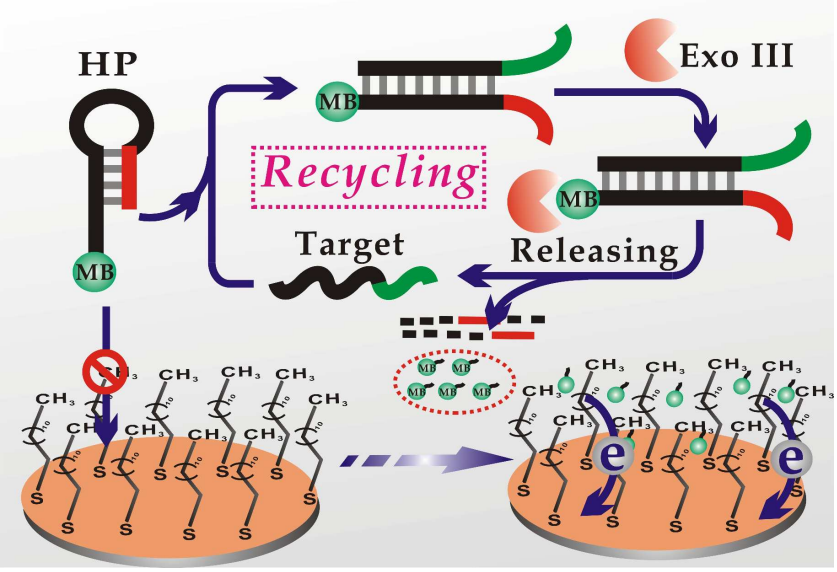

Figure 1

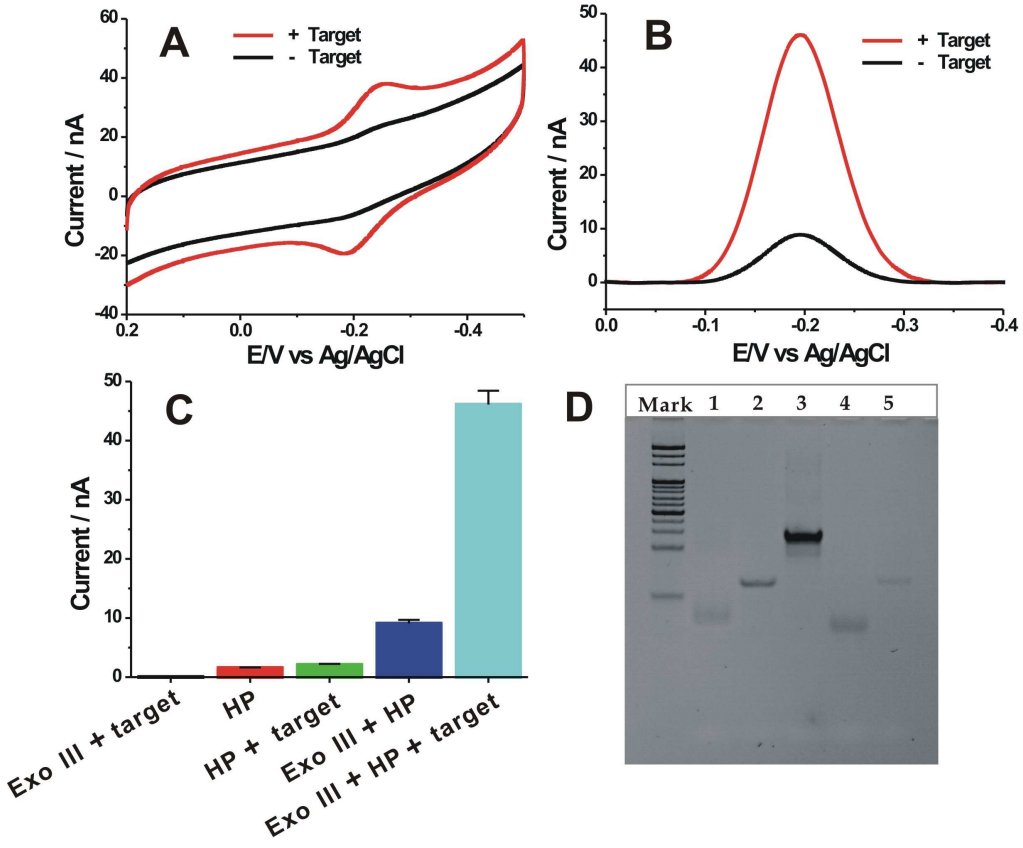
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Figure 2

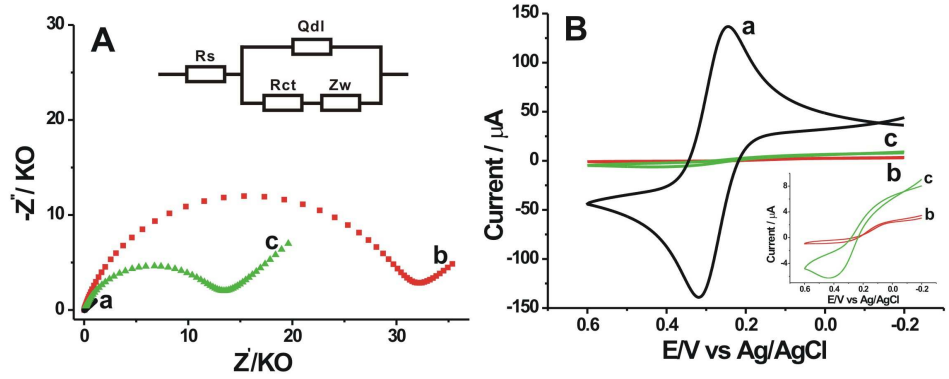

Figure 3

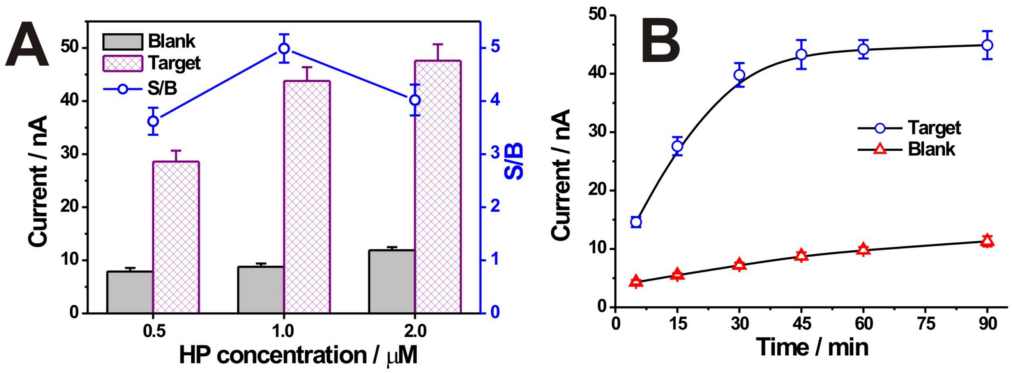
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Figure 4

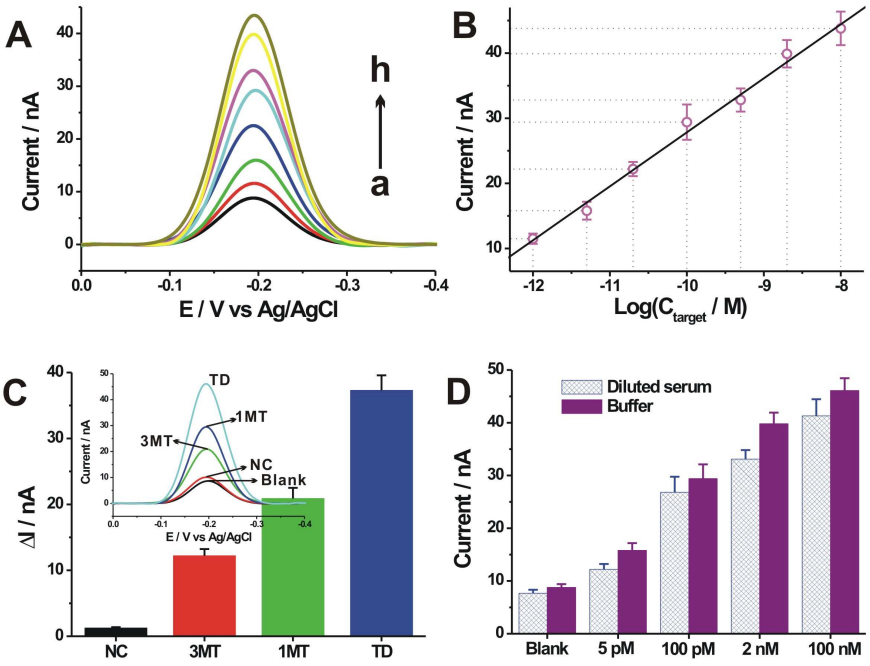
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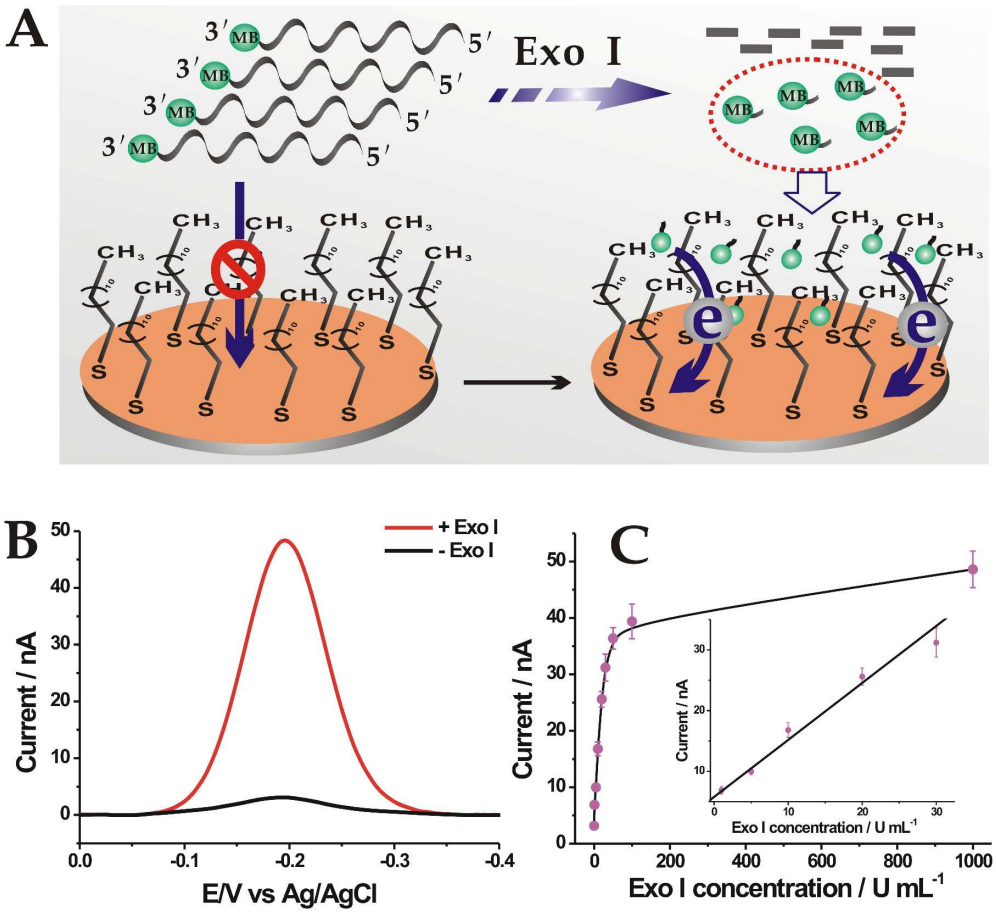
Figure 5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Exo I
B
+ Exo I
- Exo I
Current / nA
E/V vs Ag/AgCl
C
Current / nA
Exo I concentration / U mL-1

Figure 6

For TOC only