

Detection of Epidermal Growth Factor Receptor Gene Status *via* a DNA Electrochemical Biosensor Based on Lambda Exonuclease-assisted Signal Amplification

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Based on our previous work, we have constructed a new electrochemical biosensor to detect epidermal growth factor receptor (EGFR) gene mutation, which was a significant therapeutic effect predictor of target drugs for non-small cell lung cancer. In order to lower the detection limit to detect the small amount of EGFR gene status, we have employed lambda exonuclease (λ -Exo) to form a hybridization-digestion cycle. The reaction stages are depicted as follows: the target DNA hybridized with auxiliary DNA which had been modified with the λ -Exo recognition site; then, the double strands were cleaved by λ -Exo. The target DNA was released completely, and continued to hybridize with remaining auxiliary DNA, which formed a recycle for target reutilization. Finally, we detected the remaining auxiliary DNA to evaluate the amount or status of the EGFR gene. The reutilization of target DNA will help to achieve an enlarged signal with a small amount of target DNA, and the detection limit of the biosensor decreased down to 10 pM. Meanwhile, our assay can differentiate wild genes from the mutational gene of EGFR with excellent specificity. Our signal amplification method provides a research foundation for the detection system of the electrochemical biosensor by employing exonuclease, and impels the biosensor to be developed as a suitable method for EGFR detection in clinical applications.

Keywords DNA electrochemical biosensor, epidermal growth factor receptor, lambda exonuclease, signal amplification, hybridization-digestion cycles

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Introduction

Lung cancer has a relatively high incidence and mortality.¹ Tyrosine kinase inhibitors targeting the epidermal growth factor receptor (EGFR) exert a significant therapeutic effect on lung cancer. The status of the EGFR gene is an important therapeutic effect predictor for target drug treatments, which show greater efficiencies in patients with EGFR gene mutations.² The United States National Comprehensive Cancer Network (US NCCN) Guidelines recommend testing for EGFR mutations in patients with advanced lung cancer to guide the use of targeted therapies. Tumor tissue specimens used in conventional genetic testing are difficult to obtain from some patients because of the invasive nature of sample collection and the difficulties in retrieving specimens. In contrast, body fluid specimens, such as blood and tissue fluids, can be obtained easily. But the low levels of tumor DNA present in these samples increase the difficulty of detection, which puts forward new challenges for detection methods. Therefore, it is urgent to develop a highly sensitive detection method to meet the current clinical needs.

Recently, using the electrochemical method to detect human biological substances by electrochemical methods has become a hot topic.³ Electrochemical sensors combined with DNA exonuclease attract more attention with high sensitiveness for gene detection in the biosensor research field. Currently, the use of exonuclease will facilitate to form target gene recycling in order to lower the detection limit of DNA electrochemical sensors.⁴⁻⁶ In those studies, Exonuclease III, Exonuclease I and lambda exonuclease (λ -Exo) *et al.* are commonly used. λ -Exo exhibits advantages of excellent specificity and high cleavage activity for double-stranded DNA.⁷ In our previous work, we employed λ -Exo to digest double-stranded DNA to form single-stranded DNA with high efficiency.⁸ Basing on sufficient familiarity with λ -Exo characteristics, we tried to combine λ -Exo with DNA electrochemical biosensor to construct a novel electrochemical biosensor.

We also optimized the detection system to further amplify the signals based on the observation that the electron-transfer capacity is affected by the distance between electroactive materials and the electrode surface. The electron-transfer capacity is affected by the distance between the electroactive materials and the electrode-surface to some extent, with a shorter distance facilitating the diffusion of electroactive materials to the electrode surface⁹ and potentially generating a more substantial signal. In conventional methods, biotin is

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Table 1 Base sequences of the capture probe, reporter probe, auxiliary DNA and various target oligonucleotide sequences

DNA		Length/nt	Oligonucleotide sequence (5'-3')
Capture probe	C	25	SH-ggaattaagagaagcaacatctccg
Reporter probe	R1	25	cgatg aagcctacgtgatggccagc-biotin
	R2	25	biotin-cgatg aagcctacgtgatggccagc
Auxiliary DNA	A	70	PHOS-gctggccatcacgtaggctcatcgaggatttctgttgcttccggagatgtgttcttctt aattcc
Target DNA	Wild	70	ggaattaagagaagcaacatctccgaaagccaacaaggaaatcctcgatgaagcctacgtgatggcc agc
	Del 1	70	Aacatctccgaaagccaacaaggaaatcctcgatgaagcctacgtgatggccagcgtggacaacc cccac
	Del 3	70	gaaccaacatct ccgaaagccaacaaggaaatcctcgatgaagcctacgtgatg gccagcgtgg acaac
	Del 5	70	ggaaccgaaagccaacaaggaaatcctcgatgaagcctacgtgatggccagcgtggacaaccccc acgtg

located away from the electrode surface on double-stranded DNA. The binding between biotins and streptavidin-horseradish peroxidase (HRP) catalyzes the production of electroactive materials, which must diffuse a distance equivalent to the length of the double-stranded DNA before arriving at the electrode surface. This study was conducted to develop a method to accelerate the rate of electron transfer and to improve the detection of electrical signals by shortening the distance between the electroactive materials and the electrode surface.

We previously developed an electrochemical biosensor for detecting EGFR mutations and differentiating between wild-type EGFR and mutant EGFR.⁸ Additionally, we identified the mutation types by grouping analysis.¹⁰ In this study, we combined the biosensor with a λ -Exo assisted hybridization enzymatic-digestion cycle (HEC) signal amplification system to further improve the sensitivity. We also optimized the experimental design by reducing the distance between the electroactive materials and the electrode surface to facilitate the capability of signal amplification. This work provides a research foundation for employing exonuclease to construct other signal amplification detection methods and impels the biosensor to be developed as a suitable method for EGFR detection in clinical applications.

Material and Methods

Reagents

λ -Exo and λ -Exo reaction buffers were from New England Biolabs (Ipswich, Mass, USA). Streptavidin-HRP (1 mg mL⁻¹) was purchased from Invitrogen (Carlsbad, California USA). Bovine serum albumin was obtained from Roche Diagnostics (Mannheim, Stuttgart-Hohenheim, Germany) and used without further purification. The 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Neogen K-blue low-activity substrate) was from Neogen (Lansing, MI, USA). The buffer solutions used in this study were a DNA immobilization buffer consisting of 10 mM Tris-HCl (pH 7.4), 10 mM ethylenediaminetetraacetic acid (EDTA), and 1.0 M NaCl (pH 8.0). The λ -Exo reaction buffer was employed as hybridization-digestion solutions, which contained 67 mM Glycine-KOH, 2.5 mM MgCl₂, and 50 μ g mL⁻¹ BSA (pH 9.4). All solutions were prepared with Milli-Q water (18 M Ω cm resistivity) from a Millipore system.

DNA sequence design

Human EGFR mRNA sequence information was extracted from the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>, accession number Ay588246). DNA sequences, including the capture probe (C), reporter probes (R1, R2), auxiliary DNA (A), and target DNA (wild, del1, del3, del5) (see Table 1), were designed according to the respective gene

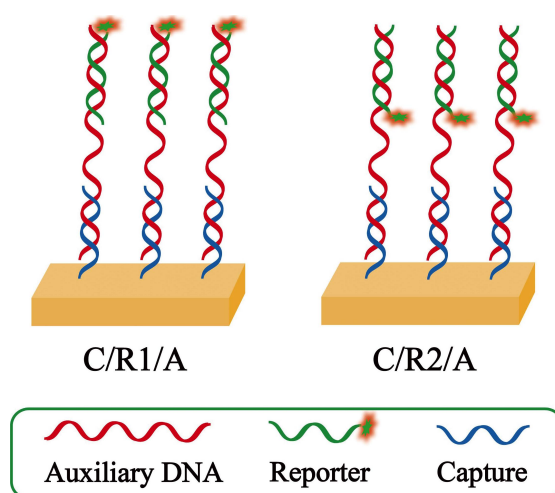


Fig. 1 Sandwich structures formed by hybridization between various probes and auxiliary strands with different biotin locations.

characteristics. C, R1 and R2 probes bind to A to form complementary double-stranded DNA that can fold into various structures. Biotins are conjugated to the terminal (away from the electrode) and middle segments of the double-stranded structures (Fig. 1, C/R1/A, C/R2/A). The location of biotins determines the distance between the electroactive materials and the electrode surface. The 5'-end of the auxiliary DNA was phosphorylated to facilitate λ -Exo recognition. The target DNA contained the wild-type sequence of the EGFR gene, which is completely complementary to the auxiliary DNA, as well as del1, del3, and del5, which are common mutations and are partly complementary to the auxiliary DNA. All oligonucleotides were synthesized and purified by TaKaRa Inc. (Dalian, China); the sequences are listed in Table 1.

DNA hybridization and digestion

The target DNA and auxiliary DNA were mixed in certain proportions ranging from 1:1 to 1:100 in a 50- μ L reaction volume containing 0.2 U μ L⁻¹ λ -Exo and reaction buffer dilution. The mixture was incubated at 37°C for 30 – 150 min. After the reaction finished, λ -Exo was inactivated at 75°C for 10 min according to the manual. The remaining auxiliary DNA was detected after incubating with capture and reporter probes.

Electrochemical biosensor detection

An electrochemical analyzer (CHI660C, Shanghai, China) was used for amperometric and current-time curve experiments, which were connected to a conventional Au disk working

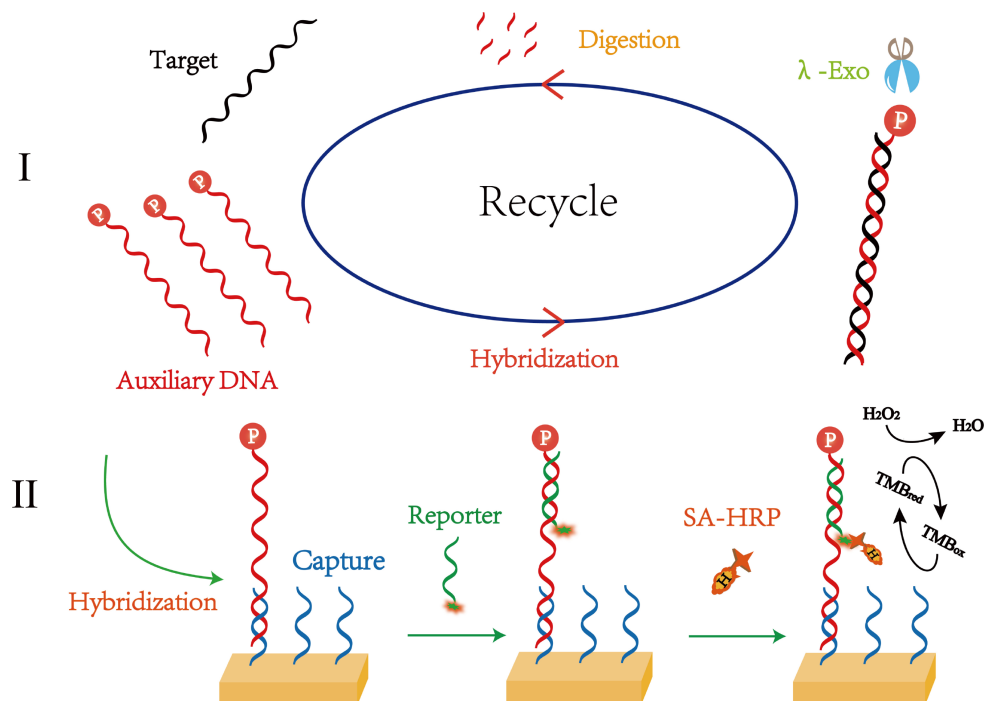


Fig. 2 Working scheme of DNA electrochemical biosensors. (I: λ -Exo selectively digested the phosphorylated strand (auxiliary DNA) of double-stranded DNA from the 5' to the 3' end, by which a hybridization enzyme-digestion cycle was formed. II: The remaining auxiliary DNA was detected by the biosensor. The auxiliary DNA hybridized with the capture probe, which immobilized on the electrode surface, and then with the biotinylated reporter probe. After streptavidin-HRP spreading on the electrode surface, HRP catalyzed the oxidation of TMB by H_2O_2 to produce a current signal, and then the biosensor was subjected to electrochemical measurements.)

electrode (2 mm diameter), an Ag/AgCl reference electrode, and a Pt wire auxiliary electrode. The BSA-based biosensor was fabricated according to a protocol developed previously.¹¹ The remaining auxiliary DNA was detected by a biosensor follow a procedure in our previous study. The auxiliary DNA was mixed with the biotinylated reporter probe (20 nM) in the hybridization buffer, and then the mixture was incubated with a capture probe immobilized on the electrode surface. After streptavidin-HRP spreading on the electrode surface, the biosensor was extensively rinsed and subjected to electrochemical measurements. Amperometric detection was performed in the TMB substrate at 0 V, and the current was sampled at 100 s. Each experiment was repeated at least three times.

Results and Discussion

Working principle

In this study, we employed the HEC cycle method for signal amplification. The λ -Exo was mixed with the target and auxiliary DNA strands in a reaction system. Hybridization of the auxiliary DNA harboring the recognition and cleavage sites of λ -Exo with the target DNA into a completely complementary double-stranded DNA, allowed λ -Exo to recognize the cleavage site and cleave the auxiliary DNA into mononucleotides. The target DNA was completely released to hybridize with the remaining auxiliary DNA for the next cycle of the HEC reaction, resulting in an HEC cycle enabling continuous digestion of the auxiliary DNA. The residual amount of auxiliary DNA was detected at the end of the reaction to reflect the number of target genes (Fig. 2). In contrast, the HEC cycle was not initiated in

the absence of complete complementarity hybridization between the auxiliary and target DNA, and then the detection of auxiliary DNA yielded higher electrical signals.

In numerous recent studies, nuclease digestion was conducted on the electrode surface. Various factors, including steric hindrance and electrostatic repulsion, as well as interactions between the active sites of enzymes and electrode surface, can reduce the rate of enzymatic digestion.^{12,13} Exonucleases have several advantages in a liquid homogeneous system, such as a high digestion rate, high selectivity, and simplicity in DNA detection. Therefore, the HEC reaction cycle in this study was carried out in a liquid homogeneous medium, followed by the detection of auxiliary DNA to indirectly determine the amount of target DNA.

Enzymatic digestion by λ -Exo

We employed λ -Exo as the enzymatic tool in our biosensor detection system. Auxiliary DNA and target DNA were mixed at a 10:1 ratio, followed by the addition of λ -Exo to a final concentration of $0.05 \text{ U } \mu\text{L}^{-1}$. This reaction mixture was incubated at 37°C in a water bath for 1 h to detect the enzymatic digestion products *via* the time-current curve method. The results were compared to those of the enzyme-free group. The results (Fig. 3) revealed no difference in the electrical signals with or without target DNA in the absence of λ -Exo. After adding λ -Exo, the electrical signals in the λ -Exo group were similar to those of the λ -Exo-free group in the absence of target DNA, which demonstrated that λ -Exo did not affect the working procedure of the detection system. However, the electrical signals decreased significantly in the presence of target DNA, indicating the involvement of λ -Exo in the HEC cycle with

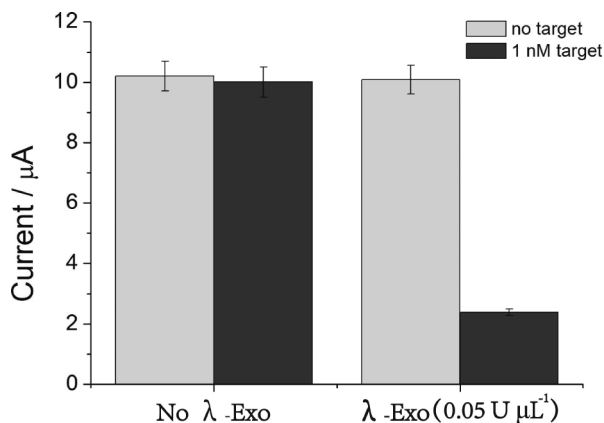


Fig. 3 Electrical signals obtained before and after enzymatic digestion in the presence and absence of target DNA. The concentration of auxiliary DNA was 10 nM. The concentration of target DNA was 1 nM.

relatively higher activity levels in this homogeneous reaction system.

λ-Exo began cleaving DNA in a 5'-to-3' direction through specific recognition and binding to the phosphorylated 5'-end of the double-stranded DNA. λ-Exo also shows some cleavage activity towards single-stranded DNA, but with a much lower activity level than that towards double-stranded DNA.⁷ Furthermore, the λ-Exo recognition site can be directly added to the double-stranded DNA by modifying the PCR primers. It also shows high enzymatic cleavage activity, with enzymatic cleavage activity per unit showing a value 10-fold that of exonuclease III. Based on these results, λ-Exo is a suitable enzymatic tool for our detection system.

Optimization of the concentration of λ-Exo

The efficiency of λ-Exo in the enzymatic digestion of double-stranded DNA is high. Theoretically, one unit of λ-Exo can release 12 mononucleotides per second. However, its cleavage efficiency varies greatly among different DNA sequences.^{14,15} Enzymatic digestion can be terminated or attenuated by specific DNA structures and sequences.¹⁶ Therefore, the optimal reaction concentration of λ-Exo must be confirmed. In the absence of target DNA, the electrical signals of the auxiliary strand-containing reaction solution did not change significantly in the absence or presence of λ-Exo at different concentrations (0.01 – 0.4 U μL⁻¹), suggesting that λ-Exo did not affect DNA hybridization in the reaction system. However, the electrical signals began decreasing when the concentration of λ-Exo exceeded 0.3 U μL⁻¹. The decreases in the electrical signals may be attributed to nonspecific cleavage because of the high concentration of λ-Exo. The electrical signals decreased dramatically after adding the wild-type EGFR gene as the target DNA to the reaction solution because of triggering HEC by λ-Exo. The electrical signals reduced significantly as the concentration of λ-Exo increased. At 0.2 U μL⁻¹, λ-Exo reduced the electrical signals to 357.3 nA, which was approximately 25% of that in the target-free group. The electrical signals did not decrease significantly as the λ-Exo concentration increased. Similar to that in the target-free group, the decrease in the electrical signals when the concentration of λ-Exo exceeded 0.3 U μL⁻¹ may be attributed to nonspecific cleavage. Our results indicate that 0.2 U μL⁻¹ of λ-Exo is the optimal concentration for the HEC reaction.

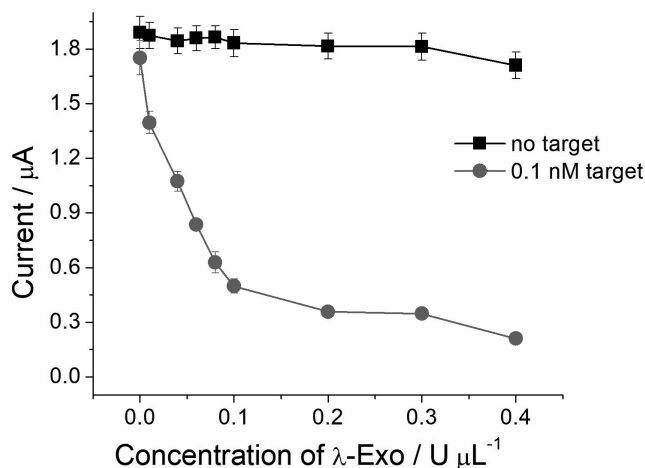


Fig. 4 Electrical signals obtained from enzymatic digestion with λ-Exo at different concentrations in the presence or absence of target DNA. The target DNA was 0.1 nM of wild-type EGFR. The concentration of auxiliary DNA was 1 nM. The concentration range of λ-Exo was 0.01 – 0.4 U μL⁻¹.

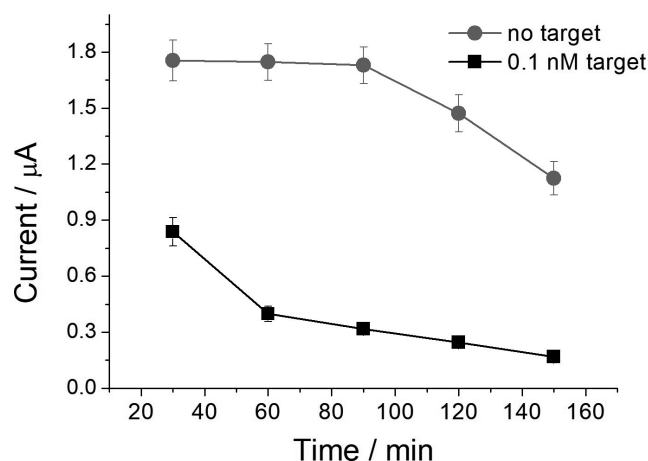


Fig. 5 Effects of different HEC reaction times on the detection of electrical signals. The reaction time for HEC was 30, 60, 90, 120, 150 min.

Optimization of HEC reaction time

A longer reaction time increases the number of HEC cycles, but may result in nonspecific cleavage. In this study, we experimentally investigated the effect of the HEC reaction time on the detection of electrical signals to determine the balance between the two variables. The results showed that the electrical signals began to decrease after being reacted with 0.2 U μL⁻¹ of λ-Exo for approximately 90 min in the absence of target DNA, indicating the presence of nonspecific cleavage by λ-Exo. The electrical signals decreased over time in the presence of target DNA because of the HEC cycle. A reaction time of 90 min led to an approximately 80% decrease in the electrical signals. However, the further reduced electrical signals after 90 min may be attributed to nonspecific cleavage by λ-Exo. Therefore, we used 90 min as the optimal reaction time in this study.

Impact of distance between electroactive materials and the electrode surface on the electrical signals

The electrical signals of the C/R1/A and C/R2/A sandwich

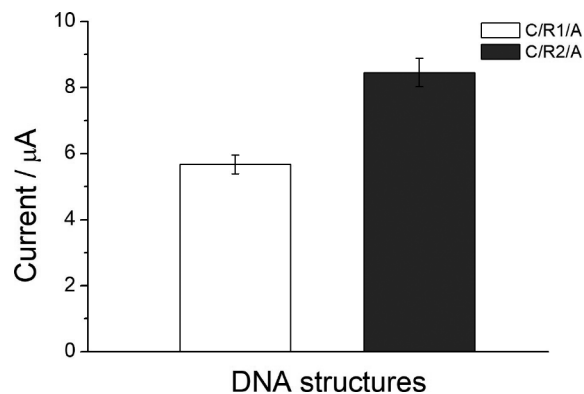


Fig. 6 Electrical signals generated with biotin-conjugated at different locations to the double-stranded DNA on electrodes. DNA structures used in the reaction include C/R1/A (left) and C/R2/A (right).

structures (Fig. 1), designed with different probes and locations of biotin, were compared. The results (Fig. 6) showed that the hybridization product, C/R2/A, yielded higher electrical signals than that of the conventional structure (C/R1/A) in which biotin is conjugated away from the electrodes. Previous studies showed that the electrochemical signal is affected by the distance between the electroactive materials and the electrode surface. Fan *et al.* manipulated the distance between ferrocene and the electrode surface to detect DNA.¹⁷ Xiao *et al.* examined the changes in the electron transfer efficiency caused by changes in the distance between methylene blue molecules and the electrode surface to detect hybridization.¹⁸ In this study, the current was produced by electroactive materials, bisazobenzidine, which originate from the HRP-catalyzed oxidation of TMB by H_2O_2 , following the binding of biotin to streptavidin-HRP. The electron-transfer efficiency increased along with the distance between bisazobenzidine and the gold electrode surface decreasing, and electrical signals increased subsequently.

Detection of synthetic segment of wild-type EGFR gene

The reaction was carried out under the optimal conditions with different concentrations of target DNA, *i.e.*, wild-type EGFR DNA (0.01 – 1 nM), which the ratio of target-to-auxiliary DNA ranging from 1:100 to 1:1. The results showed that the electrical signals decreased significantly as the concentration of target DNA increased and the ratio of target-to-auxiliary DNA decreased. The decrease in the electrical signals was particularly significant when the concentration of target DNA was <0.1 nM and the ratio of target to auxiliary DNA was >1:10. A greater number of HEC cycles in the reaction solution led to a lower amount of residual auxiliary DNA, and thus lower electrical signals. However, the decreasing trend in the electrical signals was decelerated by an increase in the concentration of target DNA, because the reduction of the ratio of target-to-auxiliary DNA limited the number of HEC cycles and prevented a further reduction of the electrical signals. Additionally, the presence of target DNA at relatively higher concentrations may influence the detection of residual auxiliary DNA. Therefore, the electrochemistry biosensor in this study is advantageous for detecting target DNA at low concentrations. The detection limit (defined as $S/N = 3$) was 10 pM, which was lower than our previous methods. Additionally, the concentration ratio of target-to-auxiliary DNA of >1:10 is conducive to the reaction.

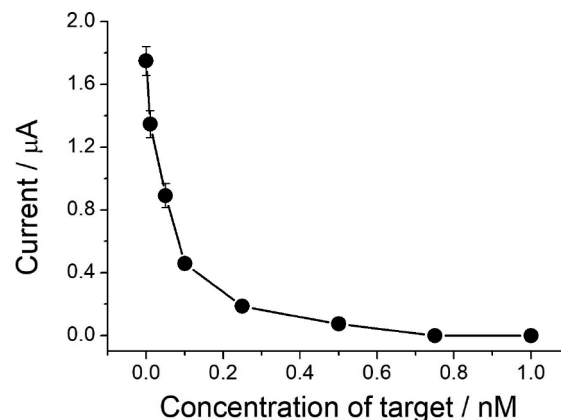


Fig. 7 Detection of electrical signals at different concentrations of synthetic wild-type EGFR DNA. The reaction was carried out with the concentration of auxiliary DNA 1 nM and different concentrations of wild-type EGFR DNA (0.01 – 1 nM). The ratio of wild-type EGFR DNA to auxiliary DNA ranged from 1:100 to 1:1.

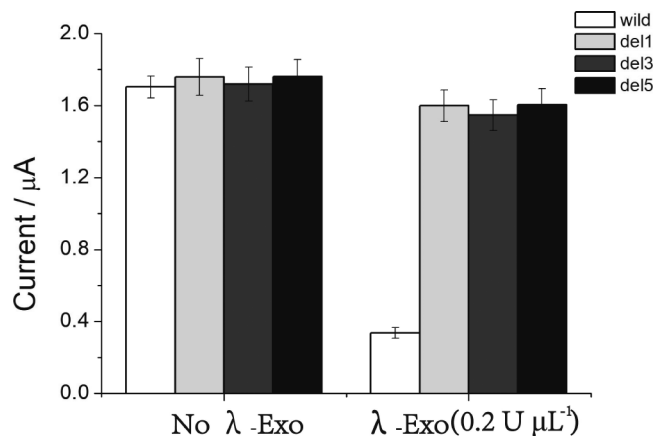


Fig. 8 Histogram depicting the detection of electrical signals generated from different types of EGFR DNA (left to right columns: wild-type, del1, del3, and del5, respectively) using electrochemical DNA biosensors. The concentration of auxiliary DNA was 1 nM, and the concentrations of wild-type, del1, del3, and del5 were 0.1 nM respectively.

Distinguishing wild-type DNA from deletion mutants of EGFR using biosensors

To evaluate the specificity of this biosensor, wild-type EGFR DNA (which is fully complementary to the auxiliary DNA) and mutant EGFR DNA with three different types of deletion mutations were assayed under the same conditions. The target DNA, including wild-type DNA, del1, del3, and del5, were detected at a final concentration of 0.1 nM each with the ratio of 1:10 to auxiliary DNA, respectively. In the absence of λ -Exo, there was no significant difference between wild-type and mutant DNAs. Whereas in the presence of λ -Exo, the electrical signals of the wild-type group were significantly lower than that of the deletion mutant groups (equivalent to only 26.7% of the deletion mutant groups), which showed high specificity of our biosensor. The complete complementarily hybridization between the wild-type EGFR DNA and auxiliary DNA favored the process of HEC cycles, and thus significantly reduced the electrical signals. In contrast, hybridization between the

deletion mutant and auxiliary DNA was rather weak because of the imperfect complementarity. Furthermore, even partial hybridization between the deletion mutant and auxiliary DNA was formed, which the absence of double strands at the phosphorylated 5'-end due to the deletion mutation located in 5'-end, significantly lowered the recognition and enzymatic cleavage ability of λ -Exo as well as the efficiency of HEC cycles.

Conclusions

This study combined "BSA-based probe carrier electrochemical DNA biosensors" with an exonuclease-assisted HEC signal-amplification system to construct a complex system with an amplifying effect achieved by enzymatic digestion in a liquid homogeneous medium. Hybridization between the target and auxiliary DNA into a double-stranded structure initiated the recognition and enzymatic digestion of the auxiliary DNA by λ -Exo, resulting in HEC cycles that allowed signal amplification through repeated recycling of a small amount of target DNA. We also optimized other conditions, such as the HEC reaction time and the λ -Exo concentration. Additionally, we examined the effect of the distance between the electroactive materials and the electrode surface on the electrical signals. Our results showed that the decreased distance between the electroactive materials and electrode surface resulted in greater electrical signals, thereby amplifying the detection signal. The biosensor constructed in this study showed a lower detection limit (10 pM for wild-type EGFR DNA) than the system using bovine serum albumin alone. Our biosensor also showed excellent specificity and could distinguish between wild-type and deletion mutants of EGFR. In conclusion, we constructed a signal-amplification detection system successfully, which enables detection and the ability to evaluate the status of trace EGFR DNA by using electrochemical DNA biosensors. Compared with traditional methods, the proposed biosensor detection method exhibits advantages, such as being time-saving, economic, and also coving high selectivity *et al.*, which will urge this assay to be a promising method for potential use in medical diagnosis for lung cancer patients. Furthermore, these strategies are expected to have further extensive applications in other DNA detection.

Conflict of Interest

There are no conflicts to declare.

Acknowledgments

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