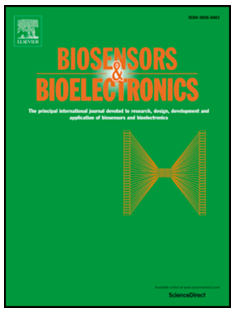

An integrated electrochemical biosensor based on target-triggered strand displacement amplification and “four-way” DNA junction towards ultrasensitive detection of PIK3CA gene mutation

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The contribution of each author to this work as following:

Tong Wang and Bin Guo (design of this work);

Qiling Peng and Decai Zhang (writing and revision);

Haiying Que, Min zhao and Haiping Wu (drawing);

Yurong Yan (discussion, supervision, funding acquisition, administration)

Yours sincerely,

Yurong Yan

An integrated electrochemical biosensor based on target-triggered strand displacement amplification and "four-way" DNA junction towards ultrasensitive detection of PIK3CA gene mutation

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Abstract

A novel electrochemical biosensor was constructed for specific and ultrasensitive detection of PIK3CA^{H1047R} gene mutation based on NsbI restriction enzyme-mediated strand displacement amplification (NsbI-SDA) and four-way DNA junction for the first time. In this biosensor, the NsbI restriction enzyme combined with strand displacement amplification (SDA) was able to specifically distinguish PIK3CA^{H1047R} gene mutation and increase the number of DNA copies to improve electrochemical response. In the presence of target mutation gene, DNA fragments produced by the cleavage event of NsbI restriction enzyme could trigger the SDA reaction to generate massive linker chains. When the linker chains were captured on the electrode, the four-way DNA junction was then attached at the end of linker chain. By integrating electroactive molecules of methylene blue (MB) into four-way DNA junction, this sandwich-like electrochemical biosensor was able to determine the specific distinction of target mutation gene with a low detection limit of 0.001%. Finally, this strategy could be used to analyze mutation gene spiked into human serum samples, indicating the potential application in genetic analysis and clinical disease diagnosis.

Keywords: PIK3CA gene mutation; NsbI-SDA; Four-way DNA junction; Methylene blue; Electrochemical biosensor.

1. Introduction

PIK3CA (phosphoinositide-3-kinase, catalytic alpha polypeptide) gene mutation is one of the most frequently mutated genes in human cancers, and has been identified in breast cancer, colorectal cancer, cervical cancer, endometrial cancer, ovarian cancer and so on (Board et al., 2010; Janku et al., 2011; Janku et al., 2012). Furthermore, PIK3CA mutations are related with poor survival in cancer patients. Among PIK3CA mutations, $PIK3CA^{H1047R}$ gene mutation is associated with early diagnosis, individualized therapy and prognosis of cancers (Kalinsky et al., 2009; Isakoff et al., 2005). Thus, it is of great importance to detect $PIK3CA^{H1047R}$ gene mutation with high specificity and sensitivity in cancer screening.

Generally, conventional methods for detecting PIK3CA gene mutations include DNA sequencing and PCR-based methods (Bachman et al., 2004; Karakas et al., 2006; Kwon et al., 2011). However, available DNA sequencing in clinic can only detect 0.1%~1% mutated DNA in the background of wild-type DNA, which challenges its widespread uptake in clinical laboratories. To improve the sensitivity of mutation assay, for example, Song's group proposed peptide nucleic acid (PNA)-mediated PCR to specifically discriminate PIK3CA gene mutation with a sensitivity of 0.2% (Zeng et al., 2017). Subsequently, Ding's group adopted an isothermal amplification strategy to analyze the same mutation with equal-level sensitivity (Shen et al., 2018). However, these methods were still practically difficult to detect small amounts of mutated alleles in a homogeneous sample. In this situation, it necessitates a higher specific and sensitive strategy for mutation analysis in clinical diagnosis.

Recently, a variety of classic strategies, such as surface ligation reaction, mismatch binding protein-mediated strategies, molecular beacon-based methods and so on, were proposed for high fidelity recognition of mutation sites (Chang et al., 2015). Indeed, these strategies can specifically detect mutation regions to avoid complicated DNA sequencing and have widely extended the mutation assay in the genetic diagnosis due to their excellent mutation discrimination capabilities. But, these methods suffer some intrinsic shortcomings such as poor specificity and low sensitivity. As an alternative biomolecule to recognize gene mutation, NsbI restriction

enzyme as a site-specific restriction enzyme can recognize specific sites (5′-TGA**G**CA) and cleave double-stranded DNA. As far as we know, the application of NsbI restriction enzyme in electrochemical biosensing for PIK3CA gene mutation detection has not been reported. Additionally, strand displacement amplification (SDA) reaction is well-known as an effective method in target recycling amplification, by which can efficiently obtain the DNA copies and amplify biological signals (Zhao et al., 2015; Li et al., 2015; Zhang et al., 2013).

On the other hand, various biosensing-based approaches such as fluorometric, colorimetric and electrochemical methods, have been utilized towards the detection of gene mutation (Guo et al., 2009; Tang et al., 2017; Oh et al., 2011; Valentini et al., 2013; Raoof et al., 2011; Esteban-Fernandez et al., 2015). Among these methods, electrochemical strategies have received increasing attention in the field of genetic diagnosis owing to its advantages of high sensitivity, low cost and rapid response (Liu et al., 2012; Hocek et al., 2011; Zhu et al., 2014). Emerging nanodevices have been introduced into electrochemical platform to further improve sensitivity including metal nanoparticles, metal-organic frameworks (MOFs), and unique DNA nanostructures (Zhu et al., 2016; Sepunaru et al., 2016; Chang et al., 2019; Chen et al., 2012; Chu et al., 2019). So far, many unique DNA nanostructures have been applied to the design of biosensors due to their ability to amplify electrochemical signal. For example, Chen and his colleagues developed an ultrasensitive electrochemical DNA biosensor using long-range self-assembled DNA nanostructures as carriers for signal amplification (Chen et al., 2012). Additionally, Zhu's group detected human telomerase RNA by a hairpin assembly-based biosensor to boost the sensitivity as low as 17.0 fM (Chu et al., 2019). As a class of novel DNA nanostructures, four-way DNA junction by simple fabrication process was chosen as candidate for developing electrochemical biosensors, by which much more electroactive molecules can be captured to produce a significant increase in electrochemical signal and boost biosensor's sensitivity (Labib et al., 2013).

Biosensing techniques have been developed to gene mutation for insistent demands of good reproducibility, short time consumption, and high sensitivity

(Ranjan et al., 2017). Inspired by the above, by integrating NsbI-SDA reaction with hairpin-based four-way DNA junction, we developed an electrochemical biosensor to ultrasensitive detect PIK3CA^{H1047R} gene mutation for the first time. By this strategy, the NsbI-SDA could not only specifically discriminate PIK3CA^{H1047R} gene mutation but also dramatically increase the number of DNA copies, while the novel four-way DNA junction could capture a large amount of electroactive methylene blue (MB) to enhance electrochemical response. More importantly, this kind of electrochemical biosensor could detect low abundance gene mutations in biological samples, suggesting potential application in both basic research and clinical diagnosis.

2. Experiment section

2.1. Materials and reagents

NsbI restriction enzyme, Klenow Fragment (KF) (3´→5´exo$^-$), Nb.BbvCI, 10 × Klenow buffer (500 mM Tris-HCl, 50 mM $MgCl_2$ and 10 mM DTT, pH 7.9) and 10 × CutSmart^{TM} buffer (20 mM Tris-acetate, 500 mM potassium acetate, 10 mM magnesium acetate and 100 μg/mL BSA, pH 7.9) were obtained from New England Biolabs (Beijing, China). GoldView Ⅰ, DNA marker and dNTP were purchased from SBS Genetech Co., Ltd (Beijing, China), TaKaRa (Dalian, China) and Thermo Fisher Scientific Inc. (Waltham Mass, USA), respectively. Tris (2-car-boxyethyl)-phosphine hydrochloride (TCEP), mercapto hexanol (MCH) and methylene blue (MB) were provided by Sangon Biotech Inc. (Shanghai, China). All oligonucleotides (HPLC grade) used in this experiment were synthesized by Sangon Biotech Inc. (Shanghai, China). The DNA sequences were illustrated in Table S1. Ultrapure water (≥ 18 MΩ, Milli-Q, Millipore) was used in all experiments.

2.2. Apparatus

Accurate oligonucleotide concentrations were evaluated by a NanoDrop 1000 spectrophotometer (Thermo Scientific Inc., Wilmington, DE, USA). The

electrophoretic gels were imaged by a ChemiDoc XRS system (Bio-Rad, Hercules, CA, UAS). DPV measurements were performed on a CHI660D Electrochemical Workstation (Shanghai Chenhua Instrument Co., Ltd., China) with a three-electrode electrochemical system (SCE as reference electrode, platinum electrode as counter electrode and gold electrode (3 mm in diameter) as working electrode). The morphology of four-way DNA junctions prepared on the mica substrate were evaluated with a SPM-9700HT atomic force microscope (AFM) (Shimadzu, Kyoto, Japan) using NSG10 single crystal silicon cantilevers (NT-MDT, Russia) with a resonant frequency of 300 kHz, force constant of 30 N/m.

2.3. Production and amplification of linker sequences by NsbI-SDA

First of all, various concentrations of mutation target (MT) DNAs, 10 nM wild-type (WT) DNAs, 20 nM complementary sequence (CS) to MT and 1 μL 10 × NEB buffer 2 were mixed together in a final volume of 10 μL to form double-stranded DNAs (dsDNAs). Then, 2 U NsbI restriction enzyme was added into the reaction system and incubated for 1 h at 37°C. The MT DNAs were completely cleaved by NsbI restriction enzyme. The reaction solution was used for the next SDA reaction. Afterwards, the SDA was reacted at 37°C for 90 min in a 10 μL reaction solution, including 1 μL cleavage products mixture, 1 nM Template, 2 U KF, 2 U Nb.BbvCI, 1 μL 10 × CutSmart™ buffer, 1 μL 10 × NEB buffer 2 and 1 μL of 250 μM dNTP (KF was used to extend DNA strands, while Nb.BbvCI was used to identify and cut specific sites on template sequence). Finally, the reaction solution was hydrated at 85 °C for 10 minutes to terminate SDA reaction. Finally, the solution was stored at 4 °C for electrochemical detection.

2.4. Construction of the proposed electrochemical biosensor

The four-way DNA junction consisted of four DNA strands, including Hairpin 1 (H1), Hairpin 2 (H2), Hairpin 3 (H3), and Quarter Sequence (QS). The secondary

structure of three hairpins was predicted by OligoAnalyzer 3.1 on line as shown in Figure S1, from which it speculated that these DNA strands could form hairpin structures very well. To form hairpin structures, H1, H2 and H3 were pretreated at 95°C for 5 min and then gradually cooled down to room temperature. All gold electrodes used in our experiments were polished with alumina powder (0.05 mm in diameter) and then sonicated in ultrapure water for ~10 min to remove impurity. Subsequently, the polished electrodes were soaked in piranha solution for 10 min to further remove the residue on the electrode surface. Then, the pretreated electrodes were modified with a 0.5 μM capture probe (CP) solution at 4 °C for ~8 h. After washing with 1 × PBS solution (pH 7.4), the CP-modified electrodes were incubated in 1 mM MCH solution at room temperature for 0.5 h to block active sites. Then, 10 μL product by NsbI-SDA was added to electrode surface and incubated at 37 °C for 0.5 h. After that, to form the four-way DNA junctions on the electrode surface, the electrodes were placed in mixture solution (containing 0.3 μM H1, 0.3 μM H2, 0.3 μM H3 and 0.3 μM QS) at 37 °C for 0.5 h, then rinsed by 1 × PBS solution. Subsequently, the electrodes were incubated in 0.5 mM MB solution (10 μL) at room temperature for 30 min in the dark. Eventually, the electrodes were washed with 1 × PBS solution to remove unbound MB for further use.

2.5. Electrochemical measurements

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were conducted in 0.5 M KNO_3 solution containing 0.5 mM $[Fe(CN)_6]^{3-/4-}$. CV curves were recorded at scan rate of 10 mV s^{-1} and EIS spectra were collected with the frequency range from 0.1 MHz to 0.01 Hz. Meanwhile, the differential pulse voltammetry (DPV) measurements were performed in the working solution (0.1 M PBS, pH 7.4) with a pulse period of 0.5 s, a pulse width of 0.2 s, a pulse amplitude of 50 mV, and potential scan from - 0.5 V to 0.0 V.

2.6. Gel electrophoresis

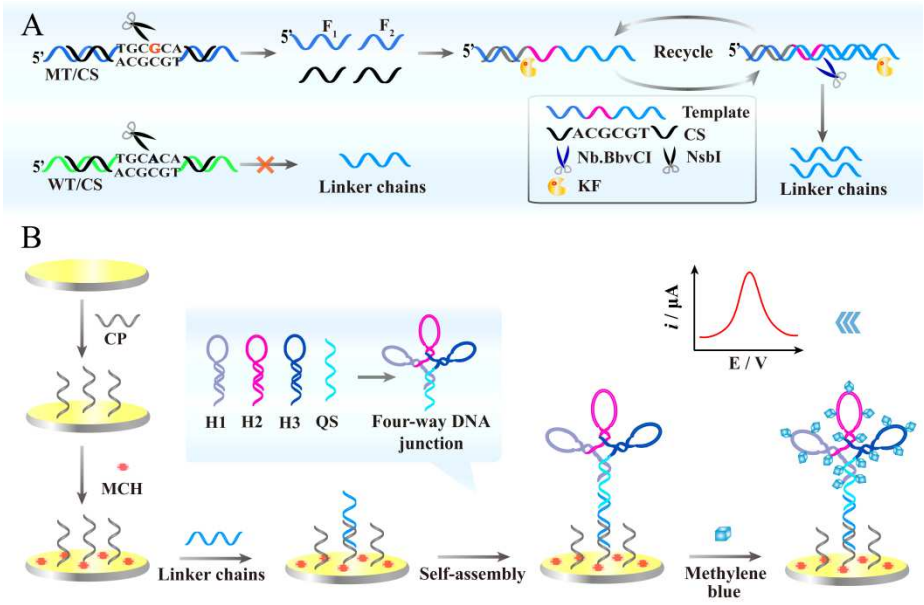
The feasibility of NsbI-SDA reaction and formation of four-way DNA junction were confirmed by 3% agarose gel electrophoresis. In a typical analysis, each sample contained 8 μL of reaction solution. The electrophoresis was performed in 1 × TBE running buffer (pH 8.3, 2 mM EDTA, 89 mM Tris, 89 mM boric acid) at 110 V constant voltages for 40 min. Then, electrophoresis images were acquired by a ChemiDoc XRS system.

3. Results and discussion

3.1. Principle of the electrochemical biosensor

As shown in Scheme 1, a sensitive electrochemical biosensor was constructed by combining NsbI-SDA and four-way DNA junction. Firstly, in part A, the CS sequence (black) perfectly hybridized to the mutation target (MT) sequence (blue) to form MT/CS dsDNA, which has a specific site (5′-TGC**G**CA). Then the site would be specifically recognized and cleaved by NsbI restriction enzyme to produce DNA fragments (F1 and F2 were fragments of the DNA fragments, they came from the MT sequence after enzymolysis). Then, F_1, one of the two DNA fragments of MT sequence, hybridized to the 3′ end of template to initiate SDA reaction with the help of Klenow Fragment and Nb.BbvCI. In the presence of dNTP, it could restart many cycles, yielding massive linker chains. However, the CS sequence hybridized with the wild type (WT) sequence (green) to form mismatched WT/CS dsDNA, in which the site (5′-TGCACA, unmutated) would not be recognized and cleaved by NsbI restriction enzyme. Thus, this NsbI-SDA reaction could not only specifically discriminate $PIK3CA^{H1047R}$ gene mutation but also dramatically increase the number of DNA copies. Subsequently, the linker chain could be captured by CP anchored on the electrode. The mixture of H1, H2, H3 and QS was dropped onto the surface of electrode to form four-way DNA junction at end of linker chain. Finally, large number of MB molecules, as electroactive indicators, could be fixed by the unique four-way DNA junction to generate electrochemical response due to its intrinsic

double-stranded stem and the G-rich ring (Chen et al., 2008; Rafiee-Pour et al., 2016). Through the NsbI-SDA method and amplification of electrochemical signal, it realized ultrasensitive detection towards PIK3CA^{H1047R} gene mutation.



Scheme 1. Schematic illustration of electrochemical biosensor for PIK3CA^{H1047R} mutation detection based on NsbI-SDA (part A) and four-way DNA junction (part B).

3.2. Characterization of the NsbI-SDA and electrochemical biosensor

To characterize the feasibility of the designed NsbI-SDA reaction, gel electrophoresis was firstly used to analyze its products in this experiment. As shown in Fig. 1A, lane 1, 2 and 3, in which each had a single band, represented WT DNAs, MT DNAs and CS, respectively. Meanwhile, the mismatched dsDNA of WT and CS also gave only one bright band even in the presence of NsbI restriction enzyme (lane 4), illustrating that this dsDNA was inactive to NsbI restriction enzyme. However, we could clearly observe multiple downstream bands in lane 5 in comparison to lane 4. In this situation, it was speculated that the matched dsDNAs of MT and CS were effectively cleaved by NsbI restriction enzyme. Additionally, some non-specific bands could be observed in lane 6 when small amounts of mismatched dsDNAs, KF, NsbI restriction enzyme, Nb.BbvCI, dNTP and template were mixed together and incubated at 37 °C for 1 h, from which it demonstrated that unwished background amplification

occurred and the mismatched dsDNAs did not take part in the SDA amplification reaction. When compared with lane 5, a downstream band could be clearly observed in lane 7, signifying massive linker chains were generated during SDA process. These results demonstrated that the NsbI-SDA reaction could effectively discriminate $PIK3CA^{H1047R}$ gene mutation and enhance its biological signal. Besides, to investigate the feasibility of four-way DNA junction's formation, we characterized the hairpin H1, H2, H3, QS, and four-way DNA junctions by gel electrophoresis analysis. As shown in Fig. 2B, lane 1, 2, 3 and 4 presented the H1, H2, H3 and QS, respectively. When they were mixed together and incubated at 37 °C for 1 h, the upstream band in lane 5 indicated successful assembly of the four-way DNA junction. And then, the morphology of four-way DNA junctions prepared on the mica substrate evaluated by AFM. Based on analysis, the structure of four-way DNA junction was similar to that of DNA tetrahedron (Li et al., 2017).

Cyclic voltammetric (CV) and electrochemical impedance spectroscopy (EIS) were employed to characterize the modified electrodes. As illustrated in Fig. 2A, it gave the CV curves of various electrodes in 0.5 mM $[Fe(CN)_6]^{3-/4-}$. It was obvious that a pair of redox peak could be observed at ~ 0.14 V and ~ 0.24 V vs SCE on the bare Au electrode (black curve). While, the successful modification of the CP probes on the bare Au electrode leaded to a decrease of electric current (red curve). When MCH, NsbI-SDA reaction product and four-way DNA junctions were added to the CP-modified electrode by turn, the electric current continued to decrease (curve c, d and e). It might be explained that biomolecular modification could block the electron-transfer between the electrolyte and electrode (Li et al., 2009; Liu et al., 2010). Meanwhile, to verify the influence of biomolecules anchored on the electrodes, EIS was carried out in 0.5 mM $[Fe(CN)_6]^{3-/4-}$ as shown in Fig. 2B. The electron-transfer resistance was exhibited by the diameter of semicircle. It was found that the biomolecules on electrode restricted the charge transfer when they were anchored on the electrode surface.

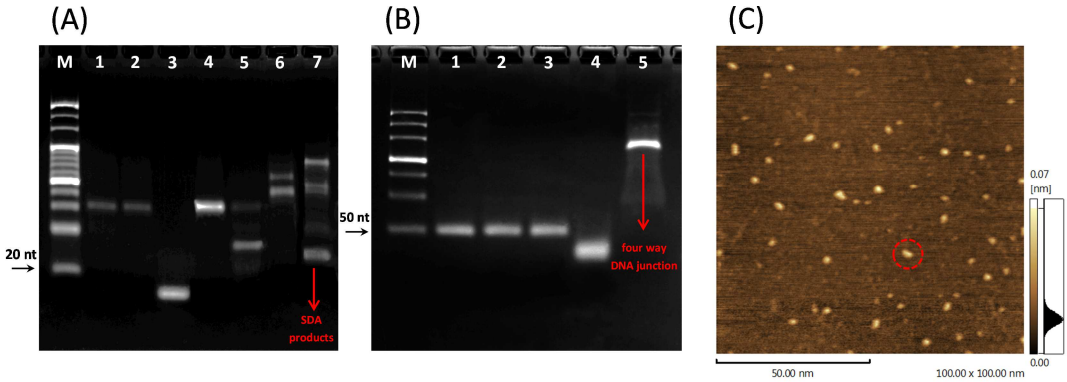


Fig. 1. Feasibility of the designed NsbI-SDA Characterized by gel electrophoresis: (A) lane M, 20-bp DNA marker; lane 1, WT; lane 2, MT; lane 3, CS; lane 4, WT/CS/NsbI restriction enzyme; lane 5, MT/CS/NsbI restriction enzyme; lane 6, WT/CS/NsbI restriction enzyme/SDA; lane 7, MT/CS/NsbI restriction enzyme/SDA; (B) lane M, 50-bp DNA marker; lane 1, H1; lane 2, H2; lane 3, H3; lane 4, QS; lane 5, H1/H2/H3/QS; (C) AFM image for four-way DNA junctions.

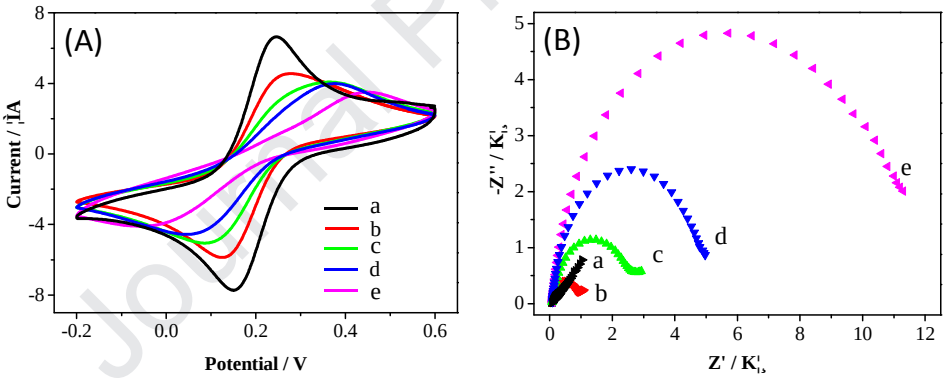


Fig. 2. The CV and EIS measurements of the modified electrodes: a) bare gold electrode. b) capture probe modified-gold electrode; c) capture probe/MCH-modified electrode: d) capture probe/MCH/NsbI-SDA reaction products-modified electrode; e) capture probe/MCH/NsbI-SDA reaction products/four-way junction DNA-modified electrode.

3.3. Electrochemical response towards detection of PIK3CA^{H1047R} gene mutation

The ultimate biomolecules-modified electrode was decorated by MB molecules, which were anchored on the four-way DNA junctions, to attain the electrochemical response. To confirm the specificity of the constructed biosensor, we evaluated it by

differential pulse voltammetry (DPV) method in 100 pM WT and MT-based solutions. First of all, we characterized the biosensor in the blank solution (The blank solution indicated that there was no mutation target (MT) DNAs or wild-type (WT) DNAs in this NsbI-SDA reaction system). Low electrochemical response was observed, which was ascribed to the electrochemical oxidations of resident MB molecules during the process of electrode preparation (Xiao et al., 2005), as shown by black curve in Fig. 3. Meanwhile, an electrochemical response on the same level was present in 100 pM WT-based solution (blue curve). When DPV measurement was performed in the 100 pM MT-based solution, a significant electrochemical response came from massive MB molecules captured by four-way DNA junctions. These results suggested that large amount of MB molecules could be fixed and had an intense electrochemical response only if mutation target (MT) was present (see Scheme 1). Obviously, the constructed biosensor has outstanding ability to specifically distinguish PIK3CA^{H1047R} gene mutation.

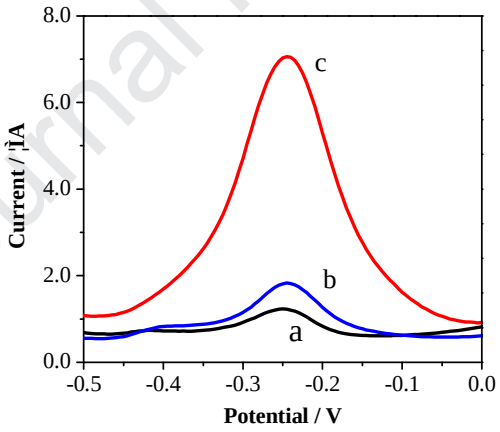


Fig. 3. The DPV responses of biosensor in: (a) blank solution; (b) 100 pM WT (wild PIK3CA^{H1047R})-based solution; (c) 100 pM MT (mutation PIK3CA^{H1047R})-based solution after NsbI-cleavage mediated strand-displacement amplification.

3.4. Optimization of experimental conditions

To obtain the best performance of the electrochemical biosensor, we optimized three important experimental parameters: NsbI cleavage time, SDA reaction time, and assemble time of four-way DNA junction. The cleavage reaction time of NsbI

restriction enzyme was firstly evaluated. As illustrated in Fig. 4A, the current responses in the DPV measurements linearly increased with cleavage time until 60 min. After 60 min, the current responses began to saturated and eventually reached to a plateau. Subsequently, we tested the influence of the SDA reaction time in the overall system. As shown in Fig. 4B, it was found that the current responses increased with the incubation time until the current responses saturated in 90 min. Then, the assemble time of four-way DNA junction was optimized. Undoubtedly, the current responses gradually increased from 15 min to 60 min, and did not change after more than 60 min, indicating that the four-way DNA junction could completely formed in 60 min. On the basis of above analysis, the results suggested that when the time of NsbI cleavage, SDA reaction, and assemble of four-way DNA junction were longer than 90 min, the electrochemical biosensor would exhibit excellent performance towards detection of PIK3CA^{H1047R} gene mutation.

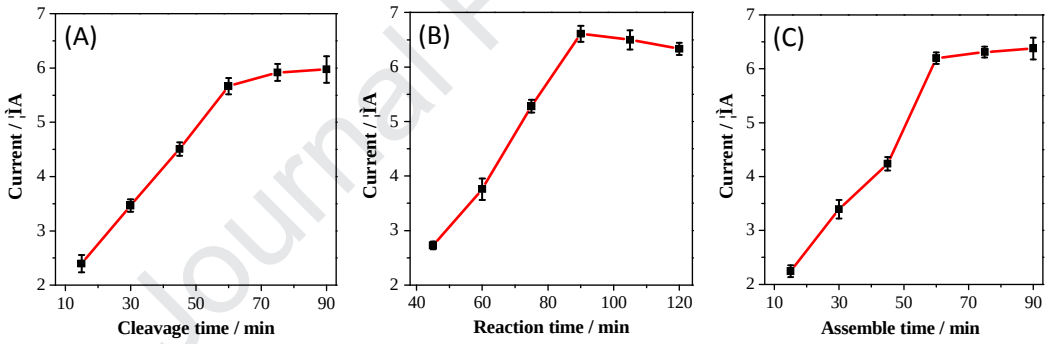


Fig. 4. Optimizations of experimental conditions: (A) NsbI cleavage time, (B) SDA reaction time and (C) assemble time of four-way DNA junction.

3.5. Biosensor's performance towards detection of PIK3CA^{H1047R} gene mutations

The electrochemical biosensor's overall performance was demonstrated in the presence of different ratios of MT to WT ranging from 10^{-5} to 0.5 (i.e. 0.001% ~ 50%). As illustrated in Fig. 5, the current response in the DPV measurements increased with MT percentage from 0 to 50.0% (Fig. 5A). The plot of current intensity vs percentage of MT showed a good linear relationship from 0.001% to 50.0% with the regression equation of $\boldsymbol{i}$ = 0.98 × lg $\boldsymbol{c}$ + 6.74 (R^2 = 0.9976) (Fig. 5B). From Fig. 5B, it was

obvious that the detection limit of this biosensor was 0.001%. Moreover, benefiting from the wonderful signal amplification capability of NsbI-SDA and four-way DNA junction, the detection limit of our biosensor was superior to other reported biosensing methods (see Table S3).

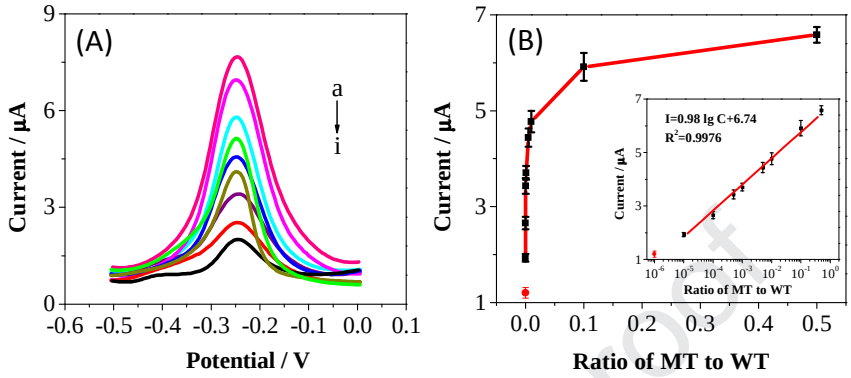


Fig. 5. (A) Current responses in the DPV measurements with the ratio of MT to WT ranging from 0 to 0.5 (i.e. 50%, 10%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.001, 0%, a→i); (B) linear relationship between current intensity and the ratio of MT to WT. Error bars represented the standard deviation of three parallel experiments.

3.6. Standard recovery test of the biosensor in spiked blood sample

To assess the applicability of the electrochemical biosensor towards detection of PIK3CA^{H1047R} gene mutation in physiological environment, the recovery test was carried out. In standard addition experiments, the ratio of MT to WT in 20-fold diluted serum was 0.01%, 1.00% and 45.00%, respectively. As illustrated in Table S2, the recovery rate of three was in the range of 96.65 ~ 103.02%, while the RSD was in the range of 4.34 ~ 5.87%. These results indicated that this proposed biosensor had potential applications for detection of PIK3CA^{H1047R} gene mutation in clinical diagnosis.

3. Conclusions

To assay PIK3CA^{H1047R} gene mutation with high specificity and sensitivity, we proposed an effective electrochemical biosensor based on the NsbI-SDA reaction and

four-way DNA junction for the first time. In this work, we have designed NsbI-SDA reaction, which could identify PIK3CA^{H107R} gene mutation and amplify biological signal in homologous systems. Then, a novel four-way DNA junction that can self-assembly directly on the electrode surface was constructed. This type of structure can bind a large number of MB molecules to improve electrochemical response to improve the biosensor's sensitivity. Compared with reported assays to gene mutation, this strategy achieved the ultrasensitive detection of PIK3CA^{H1047R} gene mutation and has been applied to complex matrices. Nevertheless, this strategy for detection of PIK3CA gene mutation is time-consuming, which affected its application in clinical diagnosis. In view of this, more efforts are needed to shorten the detection time and make it more suitable for clinical diagnosis of genetic diseases and basic researches.

Acknowledgments

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Highlights

- We combined NsbI restriction enzyme with SDA to design a new homogeneous reaction strategy, by which recognized the mutation site of PIK3CA^{H1047R} gene and improved biological signals.
- A novel four-way DNA junction by a simple and direct self-assembly process was fabricated to capture a large amount of electroactive methylene blue (MB) to enhance electrochemical response.
- A novel electrochemical sensor, based on NsbI restriction enzyme-mediated strand displacement amplification (NsbI-SDA) and hairpin-based four-way DNA junction, can determine PIK3CA^{H1047R} mutation as low as 0.001%.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: