Volume 53 | Number 20 | 11 March 2017 | Pages 2885–2992

ChemComm

Chemical Communications

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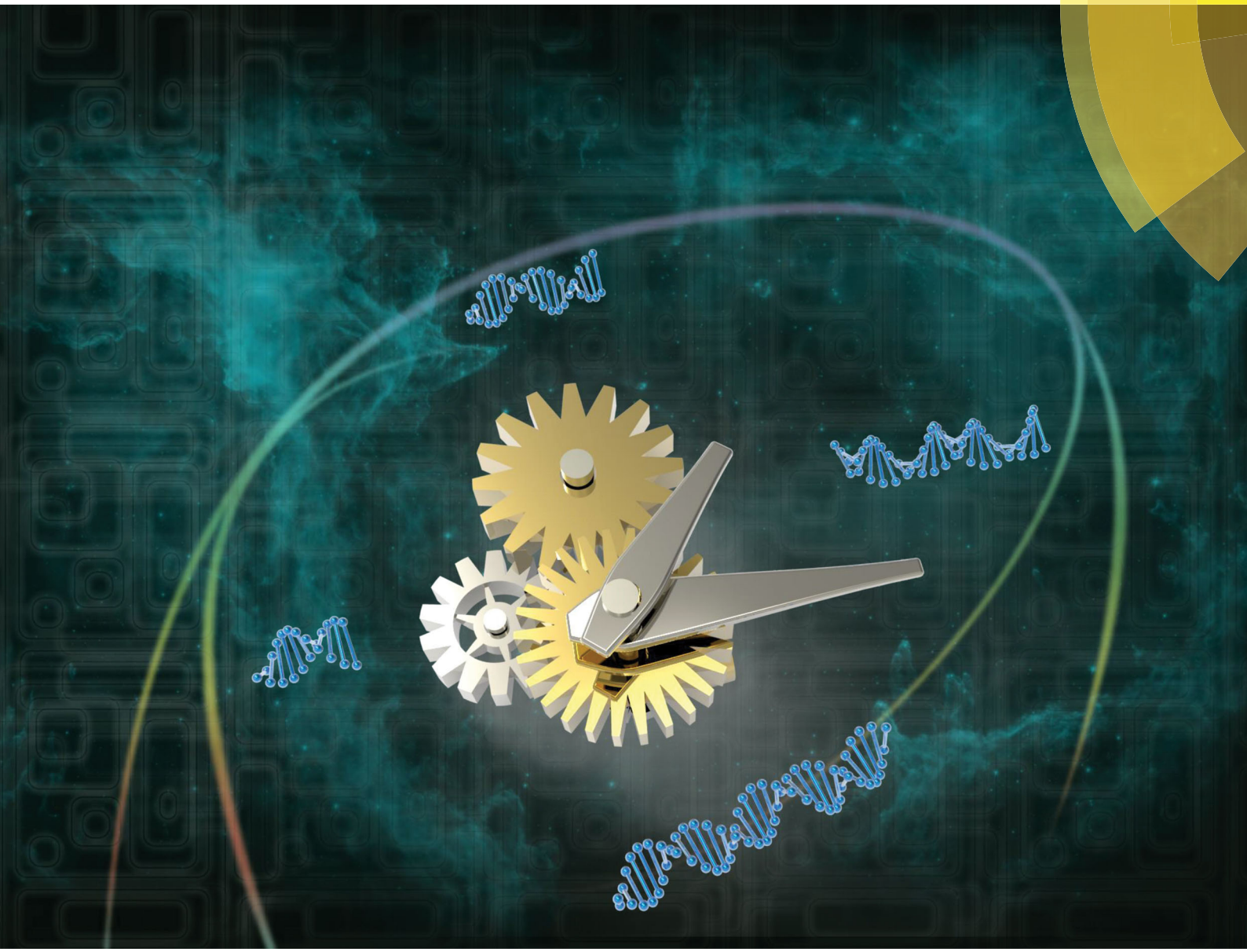

ISSN 1359-7345

ROYAL SOCIETY OF CHEMISTRY

COMMUNICATION

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ChemComm

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View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2017, 53, 2910

Received 2nd January 2017,
Accepted 26th January 2017

DOI: 10.1039/c7cc00009j

rsc.li/chemcomm

An electrochemiluminescence biosensor for Kras mutations based on locked nucleic acid functionalized DNA walkers and hyperbranched rolling circle amplification†

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Herein, an electrochemiluminescence (ECL) biosensor for ultrasensitive and specific detection of Kras mutant genes has been developed on the basis of the high discrimination capability of locked nucleic acid (LNA) and dual signal amplification techniques including DNA walkers and hyperbranched rolling circle amplification (HRCA).

Personalized medicine has important application prospects in cancer diagnosis and therapeutics, and provides more effectiveness and accuracy of the medication, reduces the side effects of the drug, and diminishes the cost of the treatment.[1] Detection of genes related to drug action can help to offer the scientific basis for individual cancer patients' medication. The Kirsten rat sarcoma viral oncogene (Kras), encoding a 21 kD Ras protein, is one of the important genetic markers for confirming the tumor targeted therapy drugs and a key downstream regulation factor in the epidermal growth factor receptor (EGFR) signal transduction pathway which is frequently activated in colorectal cancer (CRC).[2] Previous studies have revealed that only those CRC patients with wild-type Kras may benefit from anti-EGFR treatment and a poor therapeutic response to anti-EGFR antibody drugs occurs in CRC upon Kras mutations.[3] However, the Kras is mutated in 35 to 40% of CRC, which is the third most common cancer.[4] The assessment of Kras mutations is essential in prognostics and diagnostics for individualized patients' therapeutic strategies in CRC. Several methods which are currently available for detection of Kras mutations have been developed, including dideoxy sequencing[5] (regarded as the gold standard), real-time PCR,[6] high resolution melting curve analysis,[7] and pyrosequencing.[8] These methods are reliable and have relatively good sensitivity; however, much effort is still needed towards the development of selective and sensitive biosensors for Kras mutation detection.

Many signal amplification strategies have been applied to develop sensitive biosensors for DNA mutation detection.[9] The polymerase chain reaction (PCR), one of the most mature amplification techniques, has been extensively applied in the gene detection, whereas it is commonly limited by the disadvantages of complicated temperature programming, sophisticated equipment, and false positive results produced by non-specific amplification. In contrast, rolling circle amplification (RCA), an isothermal amplification technique, is much simpler, but the sensitivity of RCA is less than that of PCR.[10] Hyperbranched rolling circle amplification (HRCA) evolved from RCA has been proposed to develop biosensors for different targets with the advantages of simple processes, isothermal conditions, and much higher amplification efficiency (10^9-fold). This method was coupled with different detection strategies to develop highly sensitive biosensors for diverse targets.[11] But the selectivity of HRCA is not good, so it is still necessary to integrate HRCA with other techniques to overcome such a limitation. In our previous study, a highly selective and sensitive biosensor based on nicking endonuclease assisted target recycling and HRCA has been presented to detect the p53 DNA,[12] in which the nicking endonuclease has been employed in improving the selectivity of the biosensor. But the target DNA sequence needs to contain the specific recognition site of the endonuclease, limiting the wide amplification. Furthermore, for most HRCA based biosensors, a relatively longer reaction time is needed. Hence, it is necessary to search for some novel way to address these drawbacks.

Locked nucleic acid (LNA) has been considered as an ideal tool for single nucleotide polymorphism (SNP) detection due to its unique structural features.[13] It is a ribonucleotide derivative, containing a methylene linkage that connects the 2′-O atom with the 4′-C atom of the ribose moiety. The covalent bridge "locks" the ribose in the N-type (3-endo) conformation, and thus reduces the conformational flexibility of the ribose. This particular structure makes LNA have a higher affinity for complementary DNA or RNA sequences and a higher melting temperature (T_m) value with each LNA substitution.[14] Therefore, LNA possesses excellent mismatch recognition capacity for SNP detection. A DNA walker

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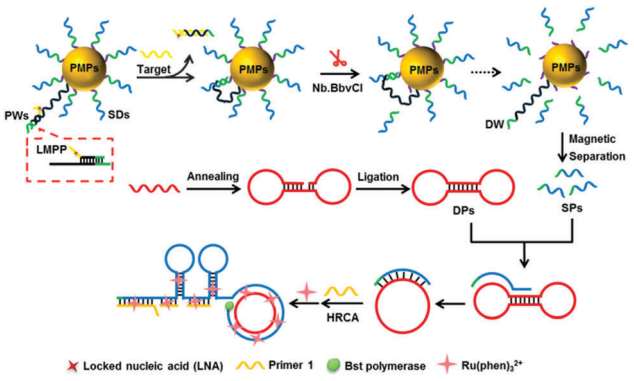
† Electronic supplementary information (ESI) available: Experimental details and data. See DOI: 10.1039/c7cc00009j

is mainly built from DNA, which is demonstrated to be a versatile and stable building block for artificial machines because of the Watson–Crick base pairing principle. The DNA walker moves along DNA tracks and realizes the rapid release of substrates within a short time, achieving a strong enhancement of the signal with high efficiency.[15]

To achieve high discrimination capability against single-base mutation and signal amplification, LNA which was used to function as a DNA walker was coupled with the high amplification efficiency of HRCA to design an electrochemiluminescence (ECL) biosensor for mutational Kras DNA sequence detection in this study. Our proposed biosensor has the advantages of high selectivity due to the capability of LNA and high sensitivity due to the dual amplification strategy of walkers through HRCA and ECL detection.[16] Such a proposed ECL biosensor was then applied to detect cancer-related mutations in Kras genes. The proposed dual signal amplification strategy enables a much broader nucleic acid analysis to dispense with specific recognition sequences in clinical cancer diagnosis and therapy.

Scheme 1 shows the scheme of the proposed biosensor. A small portion of biotin modified DNA walker tracks (DWs) was first hybridized with a moiety of an LNA modified protecting probe (LMPP) to form protected DNA walker probes (PWs), and then PWs and biotin modified substrate strand DNA (SDs) were modified on the streptavidin magnesphere paramagnetic particles (PMPs) through the specific streptavidin–biotin interaction between streptavidin modified PMPs and biotinylated DNA. The SDs contained a specific nicking cleavage sequence 5′-GC*TGAGG-3′ (* is the cleavage site), while the DWs included a recognition sequence of 5′-CCTCAGC-3′ which was complementary to the nicking cleavage sequence. The recognition site of the nicking recognition sequence was locked by the hybridization between the green part of the LMPP and the moiety of the nicking recognition sequence in the DW. The LMPP contained a DNA overhang (yellow region) and worked as a DNA toehold, in which one base was modified by LNA. The black and yellow regions of the LMPP could hybridize with the target DNA with high discrimination. Target DNA could hybridize with the LMPP and caused the release of the LMPP from the DW through a toehold-mediated strand displacement principle. Then the activated DW could hybridize with the SD to form the double stranded DNA (dsDNA) including the specific cleavage site, and hence cleaved one SD with the help of Nb.BbvCI, resulting in the release of a short probe DNA (SP) from the functionalized PMPs. Then, the free DW hybridized with a new SD and therefore triggered the next circulation of cleavage. By this means, multiple SPs were released from the PMPs. Then the residual SDs-PWs-PMPs were separated by a magnet and the solution contained multiple SPs. Simultaneously, the prepared dumbbell-shaped probes (DPs) were introduced into the solution and act as the template for the subsequent HRCA reaction. The free SPs bound with the toehold domain of DPs, causing a spontaneous strand migration and displacement that led to the formation of an activated circle. The HRCA process was initiated by the addition of Bst DNA polymerase, primer 1, dNTPs, and 10× BSA. Such HRCA reaction generated a crowd of varied length dsDNA and $Ru(phen)_3^{2+}$ intercalated into the groove of the dsDNA (acting as the ECL indicator). Then the products were added to a dialysis tube with the cutoff membrane to filtrate the redundant $Ru(phen)_3^{2+}$ for the further ECL measurement. In contrast, the DNA walker was motionless without the target DNA to start, so no SP was released. The HRCA process could not be triggered in the absence of free SPs and nearly no dsDNA was produced. The ECL intensity of the system had a direct relationship with the concentration of the target; in this context, a highly selective and sensitive ECL biosensor for target DNA can be developed.

As proof-of-principle, a mutated Kras sequence of 134A was chosen as the model target. The movement of DNA walkers was tested by using a fluorescence method and polyacrylamide gel electrophoresis (as shown in Fig. S1 in the ESI†). The results indicate that (1) the DWs can be activated by the target DNA and (2) the DNA walkers are well established. To further confirm the availability of the whole system, the resulting products were first detected by using a fluorescence technique *via* the mechanism that SYBR Green I can be embedded into dsDNA and thus yields a strong fluorescence signal. According to Fig. 1A, a low fluorescence signal was recorded when the system separately lacked the target DNA, Nb.BbvCI, DPs or Bst polymerase (curves a, b, c, d, and e), and a distinct fluorescence signal was detected in the presence of these compounds simultaneously (curve f). These results indicate that the dual amplification strategy occurs and large amounts of dsDNA are produced. In addition, the generated products of the proposed approach were also analyzed by agarose gel electrophoresis to further demonstrate its validity. As shown in Fig. 1B, no band appeared when any of the



Scheme 1 Schematic illustrating the principle of the ECL biosensor based on dual signal amplification for Kras mutant gene detection.

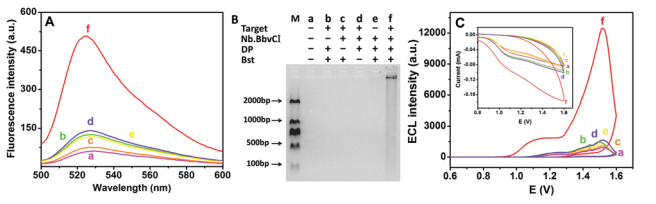


Fig. 1 (A) The fluorescence analysis of the products: (a)–(e) lacked the target DNA, Nb.BbvCI, DPs or Bst polymerase, separately; and (f) with target DNA, Nb.BbvCI, DPs and Bst polymerase. (B) Electrophoretic identification of the products by 2% agarose gel. Lanes (a–f) are as indicated above; lane M: DNA size marker. 50 fM target DNA, 10 U of Nb.BbvCI, 100 nM DPs and 8 U of Bst polymerase were used in the experiments. (C) ECL analysis of the products: curves (a–f) are in accordance with (A) (the inset figure indicates the corresponding CV curves).

four reactants (target DNA, Nb.BbvCI, DPs or Bst polymerase) was missing, indicating that the DNA walkers are latched and no HRCA reaction occurs (lanes a, b, c, d, and e). Once all the four reactants were present simultaneously, a distinct band was observed (lane f) owing to the activated DNA walker and ongoing HRCA process; the present band reveals that large amounts of varied length dsDNA are produced. The abovementioned outcomes confirm that target DNA can initiate the DNA walker and trigger the HRCA process.

Then $Ru(phen)_3^{2+}$ was intercalated into the product to test the ECL response of the system. As shown in Fig. 1C (the corresponding CV curves are shown in the inset figure), weak ECL intensity and anodic current of $Ru(phen)_3^{2+}$ oxidation were detected in the absence of Nb.BbvCI, DPs or Bst polymerase (curves a–d). When all the above three elements were present, there was no obvious change in both ECL intensity and anodic current in the absence of target DNA (curve e). Once target DNA was introduced, the ECL signal and anodic current both increased greatly. These results demonstrate that (1) the well-established DNA walker is initiated; (2) the HRCA process is triggered and hence generates numerous dsDNA segments; and (3) $Ru(phen)_3^{2+}$ can inset into the dsDNA and then acts as an ECL signal reporter.

Various experimental parameters were optimized to achieve the best sensing response of the system. The ratio of SDs and PWs covered on PMPs was first optimized because it directly affects the efficiency of DNA walkers. The dosage of SDs was set at 1500 nM and the concentration of PWs was changed from 0 to 1500 nM. As presented in Fig. 2A, the difference in ECL intensity ΔECL (ΔECL = ECL − ECL_0, where ECL and ECL_0 are the ECL intensities with and without target DNA) increased distinctly with the augmentation of PWs from 0 to 75 nM and then barely changed after 75 nM. This is probably because more PWs may influence each other owing to the steric hindrance or charge repulsion. Therefore, the optimized ratio of SDs and PWs was 20 : 1 in this study.

The concentration of the Nb.BbvCI restriction enzyme and cleavage time greatly affect the produced dosage of SPs, which further has a big influence on the ECL signal of the system. As shown in Fig. 2B, the ΔECL intensity increased remarkably with the increase of Nb.BbvCI concentration and reached a plateau after 10 U (black curve). In addition, the ΔECL intensity increased with prolonged cleavage time and reached a plateau after 10 min (red curve). Hence, 10 U and 10 min were chosen as the best conditions for the walking process in the following study.

The dumbbell probe and primer 1 are essential substances supplied for HRCA and they significantly affect the amplification efficiency, so the quantities of the dumbbell probe and primer 1 were also optimized. The black curve in Fig. 2C reveals that the ΔECL intensity increased significantly with the increase of DP concentration from 0 to 100 nM and then tended to a constant value. The ΔECL intensity also increased with the increase of primer 1 concentration first and then reached a plateau after 100 nM (red curve in Fig. 2C). These phenomena are potentially because of the steric inhibition in the limited space. So the concentrations of DPs and primer 1 were both set at 100 nM in the following study. In addition, the effect of the HRCA reaction time was also studied because it affects the produced dosage of dsDNA. As shown in Fig. 2D, the ΔECL intensity increased with prolonged reaction time and reached a constant at 90 min. Thereby, 90 min was enough for the HRCA reaction in this experiment.

Fig. 3A shows the ECL response at different target DNA concentrations under the optimized conditions. The ECL intensity increased upon increasing the target DNA. The ΔECL response had a linear relationship with the logarithm (lg) of target DNA in the range of 0.05 fM to 5 pM (inset figure in Fig. 3B). The regression equation was expressed as ΔECL = 3244 lg C_{Target} + 6170 and with a correlation coefficient of 0.996. The limit of detection was estimated to be 0.03 fM by the defined protocol of $3\sigma_b/s$, where σ_b is

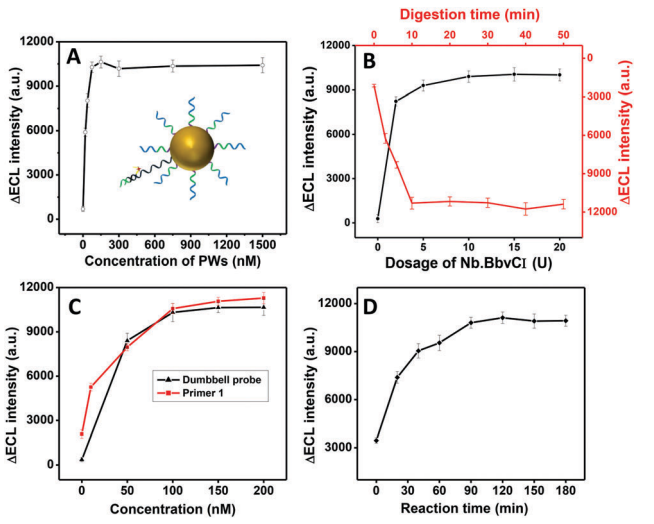


Fig. 2 Optimization of the detection conditions: (A) influence of the concentration of PWs covering PMPs. (B) Influence of the concentration and the digestion time of Nb.BbvCI. (C) Influence of the concentration of DPs and primer 1. (D) Influence of the HRCA reaction time. 50 fM target DNA was used in the experiments.

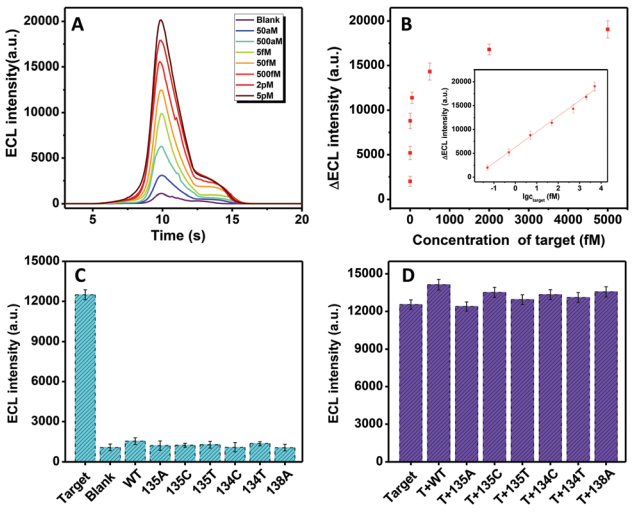


Fig. 3 (A) ECL spectra at different concentrations of the target Kras gene sequence. (B) The relationship between ΔECL and the Kras DNA sequence concentration. Inset: The calibration curve between the ΔECL and the logarithm of the Kras DNA sequence concentration. The error bars represent the standard deviation of three replicate detections. (C) Selectivity of the sensing strategy. (D) Interference study of the sensing strategy.

the standard deviation of the blank sample, and s is the slope. Compared with some other reported methods, this proposed biosensor has better sensitivity (refer to Table S2 in the ESI†). Apparently, the superior analytical performance can be attributed to the fast release of substrates of the well-designed DNA walkers and the high efficiency of HRCA for signal amplification. Furthermore, a dual signal amplification process can be achieved in 2 h, which is much quicker than those in earlier reported HRCA based biosensors for DNA sequence detection (with the reaction time over 3 h).[12,17] This is probably because the use of pre-prepared DPs as template probes of HRCA can diminish nonspecific amplification and reduce the reaction time of HRCA (1.5 h). And it can be attributed to the rapid release of substrates (SPs) of the well-designed DNA walkers within 10 min containing hybridization of the target and circulation of enzyme cleavage.

The mutated Kras genes are SNPs with seven mutations at codons 12 and 13 of two exons, which are 134A, 135A, 135C, 135T, 134C, 134T and 138A alleles. The 134A mutation was chosen as the model target DNA in this study, so other mutations and wild-type nucleic acids of Kras were used to estimate the selectivity of the proposed biosensor. According to Fig. 3C, the ECL response in the presence of 135A, 135C, 135T, 134C, 134T, and 138A, respectively (5 pM), was weak and similar to that of the blank, but the ECL response in the presence of target DNA (50 fM) was strong. These results imply that the proposed biosensor is available for discriminating the single-base mismatched DNA. Meanwhile, to assess the interference study of the proposed biosensor, the target 134A gene (50 fM) was mixed with other mutations and the wild-type gene (5 pM). There was no evident distinction between the mixture and the pure 134A (50 fM) (illustrated in Fig. 3D). These results confirm the good selectivity of this method for Kras mutation analysis again. This is probably because the LNA functionalized DNA walkers possess high discrimination capability against single-base mutation and the pre-prepared DPs can diminish nonspecific amplification of HRCA. Furthermore, the reproducibility of the strategy was explored by five repetitive experiments with 50 fM target DNA. And the relative standard deviation (RSD) was 4.8%, revealing the acceptable accuracy and good reproducibility.

To evaluate the application of the proposed biosensor, a recovery study was carried out by a standard addition of the 134A mutant sequence to the prepared normal liver cellular lysate (1.0×10^5 cells per mL). The recovery rate was defined to be the ratio between the detected concentration (measured *via* the prepared approach) and the added concentration (varied concentrations added to the cell lysate). As shown in Table S3 in the ESI,† the recovery rates were in the range of 92.7–110% and the RSDs were between 2.5% and 8.8%. These results demonstrate that the proposed method has reliable and effective application in clinical detection.

In general, a highly selective and sensitive ECL biosensor based on the high discrimination capability of LNA and dual signal amplification approaches including DNA walkers and HRCA has been proposed for the detection of Kras gene sequences. This ECL platform exhibits several attractive features: (1) facile establishment of DNA walkers—the DNA walkers are built from functionalized PMPs by some well-designed nucleic acids; (2) a fascinating distinction property—the introduced LNA provides good selectivity for discriminating the single-base mismatched DNA; and (3) ultrasensitive and faster detection. On the basis of the highly efficient DNA walkers and superior signal amplification capacity of HRCA as well as the high sensitivity of the ECL platform, the proposed ECL biosensor offers an ultrasensitive determination of Kras genes. And it also greatly reduces the detection time primarily because of the rapid release of SPs in DNA walkers (with 10 min) and convenient use of the pre-prepared DPs. Furthermore, the ECL approach has been successfully applied for the analysis of the target DNA in the cellular lysate samples with satisfactory consequences. With these advantages, our method may be employed in detecting a wide range of nucleic acids analytes without any limitation of specific sequences.

This project was partly financially supported by the National Science Foundation of China (21275031, 21575025, and 21575027), the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT15R11), and the Foundation for Scholars of Fuzhou University (No. XRC-1671).

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