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An ultrasensitive label-free electrochemical biosensor for microRNA-21 detection based on a 2′-*O*-methyl modified DNAzyme and duplex-specific nuclease assisted target recycling†

Xi Zhang,[a] Dongzhi Wu,[a] Zhijing Liu,[a] Shuxian Cai,[a] Yanping Zhao,[a] Mei Chen,[a] Yaokun Xia,[a] Chunyan Li,[a] Jing Zhang*[b] and Jinghua Chen*[a]

Based on a highly efficient 2′-*O*-methyl modified G-quadruplex-hemin DNAzyme and duplex-specific nuclease (DSN) assisted target recycling, a novel label-free electrochemical biosensor for microRNA-21 (miR-21) detection is developed here. By employing the strategy, this DNA biosensor can detect as low as 8 aM miR-21 and exhibits high discrimination ability even against a single-base mismatch.

A class of short (19–24 bases), endogenous and noncoding RNAs termed microRNAs (miRNAs) has recently been highlighted as an ideal class of tumor marker candidates for early clinical diagnosis due to their aberrant and tissue-specific expressions during the development of a variety of cancers.[1] Furthermore, compared with DNA and mRNA, miRNAs are more accurate clusters for different types of solid tumors, and the fold change in miRNAs between cancer cells and normal cells is relatively large.[1a] Therefore, it is urgently needed to detect miRNAs for early diagnosis and pathogenesis of cancers. So far, various analytical methods for the identification and quantification of miRNAs have been proposed, such as real-time quantitative PCR,[2] locked nucleic acid (LNA)-based northern blot analysis,[3] rolling circle amplification (RCA), microarray or bead-based flow-cytometry,[4] *etc*. Although a high sensitivity can be achieved, these methods still have some disadvantages including relatively long assay times, high assay costs, operational complexity and low detection accuracy.

Very recently, many isothermally exponential amplification reactions based on the cleavage of enzymes have been introduced into sensitive detection of miRNAs under a constant temperature within minutes.[5] Although these methods have brought the detection limit even down to the attomolar level, the complex and expensive enrichment or the PCR amplified procedure or the prerequisite nicking enzyme recognition site located in target DNA limits their further use for general applications. Surprisingly, Shagin *et al.* isolated a novel and stable duplex-specific nuclease (DSN).[6] It possesses considerable cleavage preference for DNA duplexes and DNA in DNA–RNA hybrids, while little activity against single-stranded DNA, or single- or double-stranded RNA. Moreover, this enzyme exhibits good ability to discriminate between perfectly and non-perfectly matched (up to one mismatch) short duplexes. Using DSN, many fluorescence signal amplification methods for miRNA detection with high sensitivity and specificity were developed.[7] Nevertheless, these methods need expensive fluorescent probes, laborious labeling techniques and sophisticated instruments, which limits their more extensive applications. Moreover, the detection limit remained in need of further improvement for practical applications.

In order to solve the limitations of labeling methods, many label-free and visual strategies based on a G-quadruplex-hemin DNAzyme were developed.[8] As one of the most interesting DNAzymes, the G-quadruplex-hemin DNAzyme has a horseradish peroxidase-like activity and can effectively catalyze the H_2O_2-mediated oxidation of several substrates accompanied by a color change. In comparison with other DNAzymes or protein enzymes, the G-quadruplex-hemin DNAzyme has obvious advantages such as low cost, easy functionalization and high stability against hydrolysis as well as heat treatment.[9] It is noteworthy that the peroxidase-like activity of this DNAzyme is highly dependent on the primary sequence and the secondary structure of the G-rich nucleic acid. Yang and co-workers demonstrated a primary sequence denoted as [B7]-3-0 that can be folded into a compact parallel G-quadruplex, and exhibits highest peroxidase activity and strong binding affinity to hemin. Furthermore, 2′-*O*-methyl modification of [B7]-3-0 can facilitate the self-assembly of the parallel structure and significantly promotes peroxidase activity.[10]

Enlightened by the above findings, we herein report a label-free electrochemical biosensor based on a 2′-*O*-methyl modified DNAzyme and DSN assisted target recycling for amplified detection

[a] *Department of Pharmaceutical Analysis, Faculty of Pharmacy, Fujian Medical University, Fuzhou, Fujian Province 350108, P. R. China. E-mail: cjh_huaxue@126.com; Fax: +86-591-22862016; Tel: +86-591-22862016*

[b] *The Ministry of Education Key Laboratory of Biopesticide and Chemical Biology, and College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou, Fujian Province 350002, P. R. China. E-mail: 1601225385@qq.com; Fax: +86-591-83769155; Tel: +86-591-83769155*

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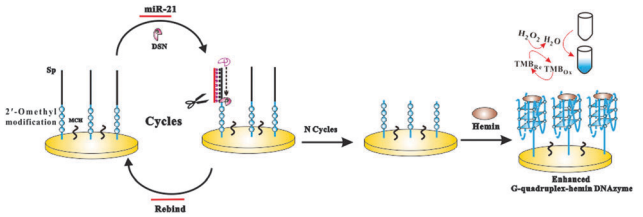

Fig. 1 The principle of the electrochemical biosensor.

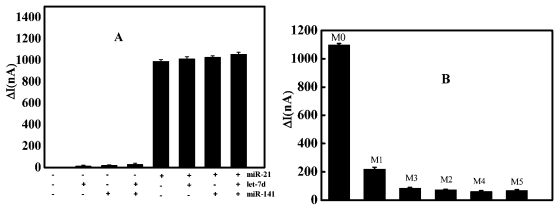

Fig. 2 Selectivity of the electrochemical biosensor. Bars represented the net amperometric signal upon different miRNAs targets. (A) miR-21 alone or with different mixtures of miRNAs(miR-141, led-7d). All miRNAs were applied at a 300 aM concentration for the assay. (B) Single-base mismatched miRNA (M1–M5) sequences. Error bars were the relative standard deviation of three independent experiments.

of miR-21, which is overexpressed in numerous solid tumor tissues in comparison to their normal counterparts.[11] The detailed principle of the sensor is illustrated in Fig. 1.

As shown in Fig. 1, the sensor contains two successive steps of isothermal enzymatic amplification. A signal probe (Sp) with a thiol group at its 5′ terminus consists of a target recognition sequence (black) and a 2′-*O*-methyl modified G-rich sequence (blue). Through S–Au bonding, Sps are covalently attached to a gold electrode (GE). In the presence of target miR-21(M), it hybridizes with a Sp to form a DNA–RNA heteroduplex. Due to the strong hydrolysis preference for DNA in DNA–RNA hybrids, DSN in turn selectively hydrolyzes the target recognition sequence of the Sp, resulting in only the 2′-*O*-methyl modified G-rich sequence part of the Sp remaining on the electrode surface. At the same time, M is dissociated from the surface of the electrode by washing and then hybridizes with a new, un-hydrolyzed Sp to initiate a new cycle. In this way, a single M is able to trigger the permanent transformation of multiple Sps to the 2′-*O*-methyl modified G-rich sequences. Ultimately, only 2′-*O*-methyl modified G-rich fragments of Sps remain on the electrode surface. Upon addition of hemin, the 2′-*O*-methyl modified G-rich sequences can bind hemin to form the peroxidase-like DNAzyme, which catalyzes the H_2O_2-mediated oxidation of TMB accompanied by a change in color of the reaction solution and an increased electrochemical current signal. However, in the absence of M, Sps cannot be hydrolyzed due to the low activity of DSN against single-stranded DNA. Therefore, Sps could not bind hemin to form the G-quadruplex DNAzyme due to the steric-hindrance effect, resulting in a poor electrochemical current signal. Using this sensing platform, a label-free, turn-on, simple, rapid, ultra-highly sensitive and selective electrochemical biosensor for miR-21 detection has been developed without miRNA labeling, enrichment or PCR amplification. It is worth noting that our proposed sensor can be easily applied to all miRNAs because DSN has no requirement for a specific recognition sequence.

By comparing circular dichroism spectra and TMB/H_2O_2 photographs of a 2′-*O*-methyl modified Sp and a Sp (see Fig. S1, ESI†), it can be concluded that 2′-*O*-methyl modification indeed facilitates the formation of perfect fine-tuned parallel G-quadruplexes and promotes peroxidase activity. Furthermore, CV signals of TMB at the DNAzyme modified GE were close to that of DNAzyme free in solution (see Fig. S2, ESI†), which indicated that the enzymatic activity of DNAzyme was not negatively affected by immobilization of the oligonucleotides on the GE surface. In addition, CV spectra of RuHex and the electrochemical impedance spectra (EIS) in the different stages of the biosensor were studied in order to confirm the preparation process. The results shown in Fig. S3 (ESI†) demonstrated that our biosensor indeed worked as we expected.

A significant challenge in the analysis of miRNAs is the ability to distinguish between different miRNA family members, which is of great importance in gaining a better understanding of the biological functions of individual miRNA. Therefore, specificity of this electrochemical strategy was firstly tested with three miRNA sequences (miR-21, miR-141 and let-7d). Different mixtures of 300 aM miR-21, miR-141, and let-7d hybridized on the GE surface with a 2′-*O*-methyl modified Sp. In the absence of any miRNA, as shown in Fig. 2A, no electrochemical signal was produced. In the presence of only miR-141, led-7d, or a mixture of them, the sensor showed only minor background signals. In the presence of mixtures of miR-21 and one or two other miRNAs mixtures, the mean signal was comparable to the electrochemical signal of only miR-21. These results suggested that there was no cross-hybridization of miR-141 or led-7d with the 2′-*O*-methyl modified Sp, and the specificities of this method were high enough to discriminate between the three family members.

The selectivity of the present biosensor in discriminating perfect complementary miRNA (M) from different single-base mismatched miRNA (M1–M5) sequences was also investigated at the same concentration (Fig. 2B). To quantitatively evaluate the discrimination ability of our sensor, we defined the discrimination factor (DF) as the ratio of the net amperometric signal (the amperometric current of target minus that of the background) obtained with M to that obtained with M1–M5. From the data shown in Fig. 2B, we found that M1–M5 have dramatically different DFs (5.0, 13.1, 15.01, 18.3 and 16.2, respectively) though they all have only a single mismatched base. These results demonstrated that a complete hybridization was not accomplished due to the single base mismatch. The high specificity of the proposed biosensor should be ascribed to the excellent capability of DSN for discriminating perfectly from non-perfectly matched DNA/RNA duplexes.

The sensitivity of the electrochemical biosensor for miR-21 detection was investigated by varying its concentration. The current signals after the sensor was hybridized with different concentrations of miR-21 and incubated in TMB/H_2O_2 at the same time were measured by amperometric techniques (see Fig. 3). The average current showed a good linear correlation with the concentration of miR-21 in the range of 50–500 aM. The regression equation is I (nA) = 2.328 C (aM) +216.3, where I is the current intensity and C is the miR-21 concentration. The regression coefficient r was 0.9920. The detection limit (3σ/S, where σ is the standard deviation of the blank solution, n = 8) was calculated to be 8 aM for miR-21.

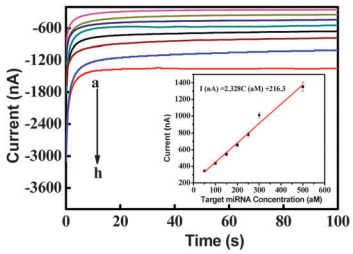

Fig. 3 Current–time curves of hybridization with different concentrations of miR-21. From a–h: 0 aM, 50 aM, 100 aM, 150 aM, 200 aM, 250 aM, 300 aM and 500 aM. Insert: a calibration curve demonstrating the peak height *versus* the miR-21 concentration.

The relative standard deviation (RSD) of 8 different sensors, which were fabricated on the same electrode for the detection of 300 aM miR-21 was 5.72%, and that of 3 different sensors fabricated on three different electrodes was 6.24%. Compared to previous methods used for miR-21 determination, the proposed electrochemical biosensor could achieve a lower detection limit (see Table S2, ESI†). Due to the extremely poor concentration of miR-21 in bio-samples, the ultra-high sensitivity of the sensor is of great significance for early diagnosis of cancers. Moreover, the biosensor requires no fluorescent labeling, which not only decreases the background signal by preventing the labeling moieties from interfering with DNA binding but also simplifies the fabrication of the sensors and lowers the cost.

In order to demonstrate the applicability of our biosensor for analyzing concentrations of miR-21 in real clinical samples, the human serum samples from 5 newly diagnosed breast cancer patients and 5 healthy donors were tested using our sensor and a commercial qRT-PCR kit as a reference. As shown in Fig. S4A (ESI†), the concentration of miR-21 in the serum from breast cancer patients was about 2.1 times higher than that detected in the serum from the healthy donors, suggesting an up-regulation of the miR-21 expression in the serum of breast cancer patients. These results were in good agreement with those reported in the literature.[12] As shown in Fig. S4B (ESI†), the results obtained using the sensor were in good agreement with those obtained by qRT-PCR of the same samples. Accordingly, the proposed electrochemical biosensor can directly measure the concentrations of miR-21 in human serum samples without pretreatment and provide a great potential in clinical diagnosis.

In short, we developed a label-free, ultra-highly sensitive and selective electrochemical biosensor based on a highly efficient 2′-*O*-methyl modified G-quadruplex-hemin DNAzyme and DSN assisted target recycling for the first time, and demonstrated its application for rapid ultrasensitive detection of miR-21 in human serum samples. Due to ultrahigh selectivity of DSN and two kinds of amplification effects due to the target recycling mechanism and 2′-*O*-methyl modified DNAzyme, the sensor can detect as low as 8 aM miR-21 and exhibits ultrahigh discrimination ability even for the detection of a single-base mismatch. These features, as well as other advantages, such as easiness of fabrication, operational convenience and low cost, make this sensor a promising candidate for early diagnosis of cancer. It must be mentioned that because DSN has no requirement for a specific recognition sequence, our sensor can be applied to all miRNAs just through rationally designing signaling probes according to the target miRNAs. Therefore, we believe that our strategy could offer a universal approach for noninvasive, early diagnosis of many kinds of cancers at the point of care.

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