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Dual-input molecular logic circuits for sensitive and simultaneous sensing of multiple microRNAs from tumor cells

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ABSTRACT

In this work, simple molecular logic circuits have been constructed by using two microRNAs (miRNAs) and the amplified fluorescent signals, respectively, as the inputs and outputs, and the application of such molecular circuits for simultaneous detection of multiple miRNA targets from tumor cells with high sensitivity has also been demonstrated. Synchronous binding of the two miRNA input targets with the fluorescently quenched double stem-loop probes leads to the unfolding of the stem-loop structure and the recovery of the fluorescence. Such fluorescence recovery is significantly enhanced via the duplex DNA fuel-assisted target recycling amplification through the toehold-mediated strand displacement reaction, thereby resulting in the realization of an "AND" gate operation and simultaneous and sensitive detection of the two miRNA targets. By assigning different inputs, an "INHIBIT" gate operation can also be achieved. In addition, the established method can be further employed to simultaneously monitor two miRNAs from low numbers of tumor cells, suggesting the potential application of the molecular circuits for disease diagnosis.

1. Introduction

Logic gates are core components of a computer for computing and logic operations. Analogously, it has been shown that a large number of logic gate operations control the metabolism, growth and reproduction of cells in biological organisms. Therefore, the construction of biomolecular logic gates has gained a great potential for bioinformatics analysis. After the DNA computation was firstly introduced by Adleman in 1994 [1], various biomolecules including nucleic acids, proteins, and small molecules have been widely used as active components for building different basic logic gate operations, such as "OR", "AND", "NAND", "XOR", "NOR" or "INHIBIT". In addition, some complex biological logic devices such as the DNA molecular devices [2], logic program photodynamic therapy [3], and cascade DNA logic programming [4] have also been established for bioanalysis applications. Despite the fact that these logic devices are still at an early stage compared to electronic circuits, they provide a great potential for the integration of target analysis and logical electronic devices, which is expected to advance the diagnosis of diseases more intelligently and accurately [5].

MicroRNAs (miRNAs), widely present in eukaryotic cells, are a class of non-coding short single-stranded RNAs with an approximate length of 22 bases and possess a wide range of functional mechanisms in the regulation of eukaryotic gene expressions [6,7]. Since miRNAs were first reported in the early 1990s [8], more than 1000 human miRNAs have been identified, enabling nearly 30% of the human genome to be targeted [9,10]. Previous studies have accumulated lots of evidences that miRNAs are involved in a number of important biological processes, including cell growth and apoptosis, insulin secretion, blood cell differentiation, and late embryonic development [11,12]. Additionally, the increased and decreased expressions of miRNAs are closely related to the occurrence of many human diseases such as malignancies [13], cancers [14], neurodegeneration [15] as well as hepatitis [16,17]. Due to the significant biological functions of miRNAs, they can be potentially used as new biomarkers for early diagnosis and prevention of diseases, as well as monitoring tumor development and prognosis of cancers [18]. However, the detection of miRNAs is largely limited by their small size, low abundance, sequence homology as well as susceptibility [19]. Therefore, the establishment of simple, fast and sensitive methods for the identification and detection of miRNAs is urgently demanded.

Several traditional detection methods, such as microarray-based techniques [20,21], Northern blotting [22], in situ hybridization [23], and bioluminescence [24] have been established for the

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analysis of miRNAs. However, they suffer from low sensitivity, unsatisfactory specificity, long assay time, poor reproducibility, and some other intrinsic limitations, which triggers further development of new detection methods by means of fluorescence [25–27], electrochemistry [28,29], electrochemiluminescence (ECL) [30,31]. Among them, the fluorescence-based approach is competitive to achieve rapid, sensitive and convenient detection of miRNAs due to the merits of simple measurement, homogeneous reaction solution, and immobilization-free of probes [32]. Early fluorescent probes for miRNA monitoring share a common characteristic that the target and the signal probe form a hybridization complex in the form of 1:1 ratio for signal generation [33]. However, the detection limits obtained from the above systems are not excellent enough for low abundance miRNAs. In order to improve the sensitivity, efforts have been committed to the establishment of sensing platforms with more efficient utilization of the target molecules. Enzyme-catalyzed target recycling, a method relying on the specific recognition and cleavage of the probe-target complex by an enzyme (usually nuclease) and subsequent release of the target for binding to another probe for signal amplification, is commonly used in biodetections, due to its high sensitivity and specificity. Nevertheless, wide application of such methods is partially limited by the requirement of harsh experimental conditions and high cost [34] with the involvement of enzymes. Therefore, the construction of enzyme-free target recycling detection approaches can potentially achieve simple and sensitive detection of miRNAs.

The majority of currently developed biosensors commonly target at a single analyte, limiting its practical application in clinical diagnosis and treatment [35]. However, in many cases, the occurrence of a disease is usually accompanied by the emergence of more than one disease markers. Many of the previously reported multifunction detection systems inevitably involve multiple signal labels and output devices [36,37], bringing high cost and complexity to the assay protocols as well as cumbersome data integration and analysis. Herein, by using two different miRNAs as the inputs, we report on the design of molecular logic circuits and its application for sensitive and simultaneous detection of miRNA-21 and miRNA-155 from cancer cells. By manipulating different inputs, "AND" and "INHIBIT" logic gate operations can be realized. Moreover, the coexistence of the two miRNA targets leads to the unfolding of the fluorescently quenched double loop-stem signal probes, and the target miRNAs are further cyclically reused with the assistance of the duplex fuel DNA to generate significantly amplified fluorescent signals for simultaneous detection of the two miRNA targets at low levels.

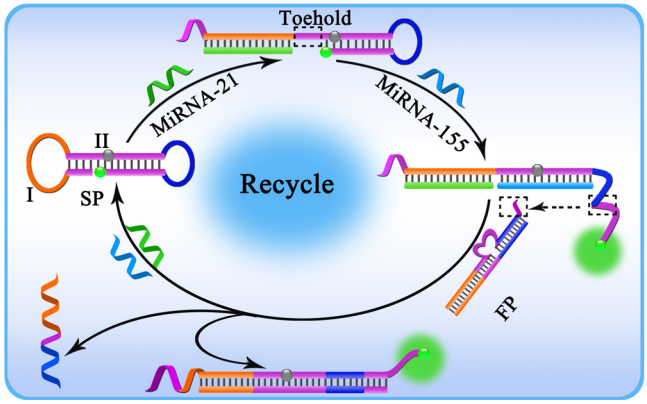
2. Experimental

2.1. Chemicals and materials

The miRNA and DNA sequences (Table 1) purified by HPLC were synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China) and Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China), respectively. Other analytical grade reagents used in the experiment were provided by Sinopharm Chemical Reagents, Co., Ltd. (Shanghai, China). All solutions involved in the experiment were prepared by ultrapure water with a resistance of 18.25 MΩ•cm.

2.2. MiRNA sensing protocol

Prior to the experiment, the FP was prepared by heating the mixture of F1 (1.2 μM) and F2 (1.2 μM) at the temperature of 90 °C for 5 min in 20 mM Tris-HCl reaction buffer (100 mM NaCl, 10 mM $MgCl_2$, pH 7.4) and then cooling down to room temperature slowly. In order to ensure the formation of hairpin structure, the same annealing procedure was applied to SP (1 μM). Next, SP (100 nM), FP (120 nM) and different concentrations of the target miRNAs were incubated at 37 °C for 75 min. After the completion of the reaction, the mixture was diluted to a volume of 200 μL to be measured. Fluorescence measurements were performed on a RF-5301PC spectrophotometer (Shimadzu, Tokyo, Japan), using a 150 W Xenon lamp (Ushio Inc, Japan) as the laser light source. The fluorescence emission spectra were obtained by scanning the fluorescence intensity in the wavelength range of 450–650 nm at the excitation wavelength of 490 nm, and the slit widths of excitation and emission were set as 5 nm.



Scheme 1. Mechanism of the dual-input molecular logic circuit sensing platform for sensitive and simultaneous detection of multiple miRNAs.

2.3. Cell culture and preparation of cell lysates

Human breast cancer cells (MDA-MB-231) and human cervical cancer cells (HeLa) were received from the Cell bank of type culture collection of the Chinese Academy of Science (Shanghai, China) and cultured in glass-bottom dishes in 5% CO_2 incubator at 37 °C through RPMI 1640 medium (Thermo Scientific Hyclone) supplemented with 100 U mL^{-1} penicilin-streptomycin and 10% fetal bovine serum. The cells were washed three times and resuspended in fresh medium for following experiments after harvesting by trypsinization, and the RNA extracts from each cell line were obtained via Trizol Reagent (Invitrogen Biotechnology Co., Ltd.). In brief, cells were lysed with the appropriate amount of Trizal and then kept at room temperature for 5 min to promote the lysis, 2-propanol was added to precipitate the cell lysate and then transferred to a microcentrifuge tube of 200 μL. Finally, the extracted solution was diluted to an appropriate concentration and stored at −20 °C for quantitative detection in real samples.

3. Results and discussion

3.1. Principle of the designed approach

The principle of our dual-input and simultaneous sensing platform for enzyme-free signal amplification analysis of multiple miRNAs is displayed in Scheme 1. The signal probe (SP), labeled with a quencher (Dabcyl) and a fluorophore (FAM), respectively, is rationally designed in a double loop–stem structure with two interrelated functional recognition segments (marked as region I and region II in Scheme 1), which are complementary to miRNA-21 and miRNA-155, respectively. SP is folded into the native and most stable configuration without the presence of the two target miRNAs. Thereby, the fluorescence signal of the system is minimized due to the close proximity of the quencher to the fluorophore. When the