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Short-probe-based duplex-specific nuclease signal amplification strategy enables imaging of endogenous microRNAs in living cells with ultrahigh specificity

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ABSTRACT

Specific nucleic acids amplification at a constant and mild temperature is important for imaging assay of endogenous microRNAs (miRNAs) in living cells. Duplex-specific nuclease (DSN) is attractive in one-step isothermal assay of miRNA; however, its intrinsic limitations of low amplification specificity and high reaction temperature greatly restrict the application scope. Herein, we present a short-probe-based DSN signal amplification (spDSNSA) strategy enabling analysis of miRNAs at body temperature with significantly high specificity. From systematic investigation of amplification reaction on different types of DNA probes, we revealed that the annealing rate between probe and target miRNA greatly affects the dynamics of amplification process. By simply shortening the length of DNA probe, the spDSNSA remarkably improved specificity without loss of amplification efficiency at 37 °C. As a proof-of-concept, let-7a was sensitively detected by spDSNSA with a limit of detection down to 30 p.M., and a specificity 10^2 – 10^4 folds higher than those of traditional DSNSA methods. The analysis of the let-7a in the lysates of A549 human lung cancer cells and BEAS-2B human lung normal bronchial epithelial cells exhibited well agreement with rt-qPCR method. Furthermore, the endogenous let-7a in A549 and BEAS-2B living cells was clearly imaged without damaging the original morphology of cells. The method provide a facile idea for extension of DNS related signal amplification strategies in the application in living cells and POCTs, and would pose a great impact on the development of simple and rapid molecular diagnostic applications for short oligonucleotides.

1. Introduction

MicroRNAs (miRNAs), regarding their key roles in regulating and controlling the expression of gene [1,2], have been deemed as important biomarkers and target molecules for biological studies and medical diagnostics [2–5]. Point-of-care testing (POCT) of miRNAs from complex media or direct imaging in living cells are greatly significant [6]. Challenged by the extremely low abundance of miRNAs, reliable analysis seriously requires efficient signal amplification techniques [7–9]. Unlike primary polymerase chain reaction (PCR)-based methods [10], homogeneous isothermal amplification assays not only free from the problem caused by sophisticated thermal cycling, but also are promising in providing sufficient amplification at constant conditions. Typical isothermal amplification strategies rely on site-specific sequence extension and nicking by polymerases and nicking enzymes, generating huge amount of desired products [11]. However, the amplification systems are usually more complicated than expected. Beyond the needs of polymerases and nicking enzymes, sophisticated triggers and templates containing specific recognition sequences for nicking enzymes are also essential [11]. These requirements increase the chance for errors and costs [12], and are discommodious in POCTs and cell imaging assays. Recently, duplex-specific nuclease (DSN) received considerable attention due to its unique substrate specificity that only hydrolyzes double-stranded DNA (dsDNA) or DNA in DNA/RNA hybrids. The possibility for cleavage of the DNA probe in probe/target hybrid is promising for target units recycling, leading to significant signal amplification. Based on linear Taqman probe, Ye *et al.* devised an elegant one-step DSN signal amplification (DSNSA) method enabling sensitive detection of 100 fM miRNA within 30 min [13]. The use of hairpin-structural probe in DSNSA further improved the assay specificity. By coupling nanomaterials, such as tungsten disulfide (WS_2) nanosheets [14], graphene oxide (GO) [15,16], and gold nanoparticles (AuNPs) [17] with DSN-mediated target recycling, miRNAs can be sensitively detected by fluorometry [13–17] or electrochemistry [18] in batch or even in fixed cells. The DSN-mediated reaction can be used to link other signal amplification formats, therefore to cascade multiple

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amplifications, like the recently reported method of triple signal amplification strategy [18].

In these elegant strategies, the core process is the DSN-mediated reaction, which actually has two intrinsic limitations. (1) Reactions on linear DNA probe exhibit low selectivity for homologous oligonucleotides even having four-base differences [19]. Although on HP can improve the specificity of DSNSA, the HP commonly needs to be specially modified for example by 2-OMe-RNA [19], which requires additional cost and complicated synthesizing procedure. Furthermore, the amplification efficiency will be reduced greatly due to the slow hybridization kinetics. (2) To strengthen the amplification power, the temperature for DSNSA always needs ~60 °C, which is obviously higher than room or body temperatures. It implies that even if the DSNSA strategy is capable of carrying out assays at a constant temperature without thermal cycler, it still requires heating instruments to maintain desirable temperature for amplification to take place efficiency. As a result, such high temperature makes the POCTs discommodious and the assays in living body almost impossible.

Aiming at overcome the limitations of DSNSA, here we present a short-probe-based DSNSA (spDSNSA) strategy. In view of the rate-determining step of DSNSA process by different types of probes, we revealed that the hybridization rate between DNA probe and target miRNA greatly affected the dynamics of DSNSA. On a short linear DNA probe, the hybridization rate was significantly increased along with remarkable improvement in specificity. The spDSNSA strategy was able to work at body temperature without loss of amplification efficiency. Taking the let-7 miRNA family as a target model, we investigated the analytical performance of the spDSNSA method in terms of the sensitivity, specificity, and the detection accuracy for cell lysates. The feasibility of the spDSNSA method for cell imaging assay was demonstrated by the analysis of intracellular let-7a in fixed or even living human lung cells.

2. Experimental

2.1. Reagents and materials

Oligonucleotides were obtained from TaKaRa Co. Ltd. (Dalian, China). Their sequences are listed in Table S1. RNase inhibitor, egg lecithin, cholesterol and DEPC-treated water were obtained from Sangon Biotech Co. Ltd. (Shanghai, China; DEPC = diethylpyrocarbonate). DSN was purchased from Evrogen Joint Stock Company (Moscow, Russia). Fetal bovine serum and the RPMI 1640 were purchased from Gibco (Carlsbad, CA, USA). All other reagents were in analytical grade. The solutions used in all experiments were prepared using DEPC treated deionized water.

2.2. Standard free energy of hybridization between PNA probe and target

Molecule structures of the bases in PNA, RNA and DNA were built and optimized by Hyperchem (8.0) and saved as PDB data format. The binding energy of the interactions between PNA and DNA, PNA and RNA, DNA and RNA were evaluated from base pairs, in which one base was set as receptor molecule and the other base as ligand molecule. Association values were obtained by Autodock Tool (1.5.6). Default arguments of grid box were adopted when receptors were docked.

2.3. Preparation of probe/DSN loaded liposome

Liposome was formulated by thin film hydration method. 35 mg egg lecithin and 11 mg cholesterol were placed in a single-neck flask and dissolved in 20 mL of absolute ethanol. The solution was rotary-evaporated using a rotary evaporator to dryness, and a thin lipid film was generated. The resultant lipid film was then hydrated by 5 mL of PBS (pH 7.4) at 37 °C for 30 min. After being fully hydrated, the mixture was agitated and treated with ultrasound until the solution was clear and transparent. The final liposomal solution was stored at 4 °C.

The loading of probes and DSN enzyme into liposome was carried as follow. 3 μM DNA probe and 1 U DSN in 1 × DSN master buffer were added to 27 μg liposome and mixed gently. The mixture was stored at −20 °C for 12 h, and dissolved and mixed at room temperature. This procedure was repeated three times before applying to visualization of miRNA in cells.

2.4. Cell culture and RNA extraction

A549 human lung cancer cells and BEAS-2B human lung normal bronchial epithelial cells were purchased from the American Type Culture Collection (ATCC). The A549 and BEAS-2B cells were cultured in DMEM medium supplemented with 10% fetal calf serum, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

Total RNA was extracted from A549 and Beas-2B cells using Trizol reagent (TAKARA, Dalian, China) following the manufacturer's instructions. Cells were added with 1 mL of trizol and vortex vigorously to a homogenous lysate. The lysate was added with 0.2 mL of chloroform and vortex for 15 s, and then incubated at 25 – 30 °C for 2 – 3 min. The resultant mixture was centrifuged at 4 °C for 15 min at 12,000 r min^{-1} to separate the aqueous and organic phase. The aqueous phase was transferred into a fresh tube, and added with equal volume of isopropyl alcohol. After incubation at 15 – 30 °C for 10 min and centrifugation at 4 °C for 10 min at 12,000 r min^{-1}, the resultant aqueous phase was discarded, and the precipitation was washed with 800 μL of 75% alcohol. The resultant precipitation was further centrifuged at 4 °C for 5 min at 7500 r min^{-1}. Abandon of the alcohol, the obtained total RNA was eluted with DEPC-treated water and kept in a refrigerator at –80 °C for further use.

2.5. Reverse transcription

The reverse transcription of the obtained total RNA was carried out in a mixture containing 12.5 μL of RNase free doubly-distilled water, 2 μL of 5 × Prime Script Buffer (for real time), 1 μL of Prime Script RT Enzyme Mix I, 0.5 μL of RT Primer (10 pmol $μL^{-1}$) and 4 μL of the extracted total RNA. The mixture was incubated at 37 °C for 40 min, and then at 85 °C for 2 min.

2.6. Quantitative rt-qPCR analysis

Quantitative real-time fluorescence analysis was performed on a 7500 Real-Time PCR System (Applied Biosystems, USA). Forty cycles of amplification were performed. Each cycle comprised an initial denaturation step at 95 °C for 2 min, a denaturation step at 93 °C for 15 s, and annealing and extension steps at 55 °C for 25 s. The detection system was 25 μL of a mixture containing 18.5 μL of doubly distilled water, 2.5 μL of 10 × PCR buffer (with SYBR Green I and ROX), 0.5 μL of dNTPs, 0.5 μL of TAQ, 2 μL of *c*DNA, 0.5 μL of forward primer and 0.5 μL of reverse primer (Table S1). The C_t values were converted into absolute let-7a copy numbers using a standard curve obtained from synthetic let-7a.

2.7. Detection of miRNA in cell lysates

Detection of miRNA was performed in a 30 μL of reaction mixture of 0.1 U DSN, 20 U RNase inhibitor, 100 nM DNA probe and different concentrations of let-7a in 1 × DSN master buffer (50 mM pH 8.0 Tris-HCl, 5 mM $MgCl_2$ and 1 mM DTT). After reaction at 37 °C for 150 min in a 7500 Real-Time PCR System (Applied Biosystems, USA), the reaction mixture was added with 30 μL of 10 mM EDTA and incubated at 37 °C for 5 min to inactive DSN enzyme. The fluorescence spectra of the resultant mixture between 505 and 600 nm were recorded on a F-7000 fluorescence spectrophotometer (Hitachi, Japan) at room temperature.