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Direct fluorescence detection of microRNA based on enzymatically engineered primer extension poly-thymine (EPEPT) reaction using copper nanoparticles as nano-dye

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ABSTRACT

A new strategy based on enzymatically engineered primer extension poly-thymine (EPEPT) and nanomaterials in situ generation technology is reported for direct detection of microRNA (miRNA) in a fluorescence turn-on format using the sequential and complementary reactions catalyzed by Klenow Fragment exo$^-$ (KFexo$^-$) and terminal deoxynucleotidyl transferase (TdTase). The short miRNA can be efficiently converted into long poly-thymine (polyT) sequences, which function as template for in situ formation of fluorescence copper nanoparticles (CuNPs) as nano-dye for detecting miRNA. The polyT-CuNPs can effectively form and emit intense red fluorescence under the 340 nm excitation. For the proof of concept, microRNA-21 (miR-21) was selected as the model target to testify this strategy as a versatile assay platform. By directly using miR-21 as the primer, the simple, rapid and sensitive miRNA detection was successfully achieved with a good linearity between 1 pM and 1 nM and a detection limit of 100 fM. Thus, the EPEPT strategy holds great potential in biochemical sensing research as an efficient and universal platform.

1. Introduction

MicroRNAs (miRNAs), which act as important post-transcriptional regulators of gene expression, play an important role in many human diseases, including cancer (Hobert, 2008; Sawyers 2008). The sensitive and selective detection of miRNAs is of great significance for better understanding biological functions of individual miRNA. Owing to its signal amplification ability, the in vitro polymerization of nucleotides has been intensely engaged in miRNA analysis, such as polymerase chain reaction (PCR) (Chen et al., 2005; Yu et al., 2013), rolling circle amplification (RCA) (Cheng et al., 2009; Zhou et al., 2010; Deng et al., 2014), and strand displacement amplification (SDA) (Zhang et al., 2016; Zhou et al., 2014). These sensors based on above methods generally utilize fluorophores or dye-labeled probes as signal reporters (Qing et al., 2014). The design and synthesis of these signal reporters are usually complicated to operate and require extensive experiments involving multiple stages and time-consuming optimization (Zhang et al., 2015; Wang et al., 2013). More importantly, these signal reporters display a certain degree of nonspecific adsorption, thus increasing their environmental susceptibility and reducing sensitivity (Qing et al., 2014).

Recently, to overcome these drawbacks, as promising candidates to conventional fluorophores, DNA-templated fluorescence copper NPs (CuNPs) with strong and robust fluorescence emission have drawn increasing attention, including random double-stranded DNA-templated CuNPs (dsDNA-CuNPs) and single-stranded poly-thymine-templated CuNPs (polyT-CuNPs) (Jia et al., 2012; Rotaru et al., 2010; Song et al., 2015; Qing et al., 2013; Liu et al., 2013). They show great potential for biochemical applications, due to their amazing features of facile synthesis, excellent photophysical properties, and good biocompatibility (Xu et al., 2014; Ma et al., 2009; Chen et al., 2012; Zhang et al., 2013; Qing et al., 2016). The dsDNA-CuNPs have already been facilely utilized to contrive versatile DNA-related detection strategies for miRNAs (Zhang et al., 2015; Wang et al., 2013). However, single-stranded poly-thymine (polyT) DNA can also be acted as an efficient template for formation of fluorescence CuNPs (Qing et al., 2013), which is strictly length-dependent (Liu et al., 2013), and more controllable. Furthermore, PolyT-CuNPs exhibit red fluorescence with large MegaStokes shifting, which is well-suited as a fluorescent probe for detection of targets from complex biological matrix, as it can enable the removal of strong background signal from complex biological systems. However, until now, the application of polyT-CuNPs for miRNA detection is still scarce.

Herein, we unveil a simple and cost-effective enzymatically

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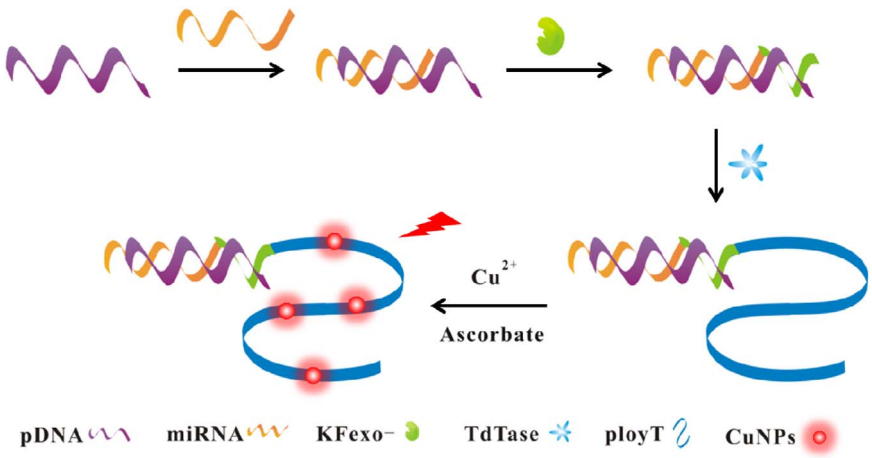

Scheme 1. Scheme showing the principle of enzymatically engineered primer extension poly-thymine (EPEPT) strategy.

engineered primer extension poly-thymine (EPEPT) strategy for the rapid and sensitive analysis of miRNAs by exploiting polyT-CuNPs nano-dye in situ synthesized. An outline of the designed procedure is illustrated in Scheme 1. First, the target (miRNA) hybridizes with the probe DNA (pDNA) to form primer-template complex. Then, polymerase Klenow Fragment exo$^-$ (KFexo$^-$) catalyzes the primer extension, generating a short DNA strand, which is complementary to pDNA (Nelson et al., 2004). In the presence of doxythymidine triphosphates (dTTPs), terminal deoxynucleotidyl transferase (TdTase) can directly catalyze 3′-OH of the short DNA strand to form polyT sequence without a template (McCaffrey et al., 1981; Fowler and Suo, 2006; Bollum 1974). After Cu^{2+} and ascorbate are added into the system, strong red-emitting CuNPs are formed on the resulting polyT sequence. Because of the affinity between thymine and Cu^{2+}, thymine-complexed Cu^{2+} was reduced to Cu° by ascorbate along the shape of polyT sequence scaffold (Qing et al., 2013). As described above, a short miRNA can be efficiently converted into a long polyT sequence, which acts as a template in the formation of fluorescence CuNPs nano-dye in situ synthesized. Furthermore, fluorescence intensity of polyT-CuNPs is positively correlated with concentration of miRNAs, which realizes detection of miRNA with low background and high sensitivity.

2. Experimental section

2.1. Reagents

All the synthetic DNA, RNA, doxythymidine triphosphates (dTTPs) and RNase inhibitor were obtained from TaKaRa Biotechnology Co. Ltd. (Dalian, China). All the oligonucleotides were purified by HPLC. Sequences of the oligos are listed in Table S1. Polymerase Klenow Fragment exo$^-$ (KFexo$^-$) and terminal deoxynucleotidyl transferase (TdTase) were purchased from New England Biolabs (NEB). 3-(N-morpholino) propanesulfonic acid (MOPS) was purchased from Dingguo Biotech (Beijing, China). Copper sulfate ($CuSO_4 \cdot 5H_2O$) and sodium ascorbate were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Human breast adenocarcinoma cell (MCF-7) and human lung adenocarcinoma epithelial cell (A549) were obtained from the second affiliated hospital of Nanchang University. DMEM high glucose medium, penicillin, streptomycin and fetal bovine serum were purchased from Thermo Scientific HyClone (MA, USA). All stock and buffer solutions were prepared using ultrapure water (18.2 MΩ cm from Millpore purification system). In RNA-related experiments, DEPC-treated deionized water was used.

2.2. Apparatus

The fluorescence measurements were performed with a Hitachi F-7000 spectrofluorimeter (Tokyo, Japan) equipped with a xenon lamp. Gel electrophoresis images were acquired with a VersaDoc 4000 imaging system (Bio-Rad, USA). Atomic force microscopy (AFM) image was recorded under the mode of ScanAsyst using Bruker MultiMode-8 at. force microscopy (Bruker, USA). The UV–vis absorption spectra were collected on a UV-2450 spectrophotometer (Shimadzu, Japan). JEOL 2010 transmission electron microscope (TEM, Japan) were used to characterize the size and morphology of the CuNPs.

2.3. Polymerization reaction

First, 1000 nM pDNA and an appropriate amount of the target miRNA in a reaction volume of 10 μL, were denatured at 80 °C for 5 min and cooled slowly to room temperature. Then, 5 U of KFexo$^-$, 20 mM Tris-Ac (pH 7.9), 50 mM KAc, 10 mM $Mg(Ac)_2$, 0.25 mM $CoCl_2$, 1000 μM dTTPs, and 9 U of TdTase in a reaction volume of 20 μL were added and incubated at 37 °C for 3 h.

2.4. Formation of PolyT-CuNPs

To prepare polyT-CuNPs, 1 mM sodium ascorbate was added into the solutions. After blending completely, 100 μM copper ion solutions was added and allowed to react for about 5 min at room temperature. The resulted polyT-CuNPs were immediately characterized or used for following experiments.

2.5. Measurement of fluorescent spectra

The fluorescence spectra were measured in a 1 × 1 cm quartz cuvette. The excitation wavelength was at 340 nm, and the spectra were recorded between 520 and 650 nm. The fluorescence emission intensity was measured at the peak wavelength of 600 nm.

2.6. Gel Electrophoresis analysis

The products used were analyzed with 1.8% (w/w) agarose gel electrophoresis and the effect of polymerase in the reaction was investigated by 20% polyacrylamide gel electrophoresis, running in 0.5 × TBE (22.5 mM Tris-Boric acid, 5 mM EDTA, pH 8.0) at room temperature. Electrophoresis was performed at a constant potential of 110 V.