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Detection of microRNA in clinical tumor samples by isothermal enzyme-free amplification and label-free graphene oxide-based SYBR Green I fluorescence platform

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ABSTRACT

MicroRNAs (miRNAs) are a kind of small molecules that involve in many important life activities. They have higher expression levels in many kinds of cancers. In this study, we developed an isothermal enzyme-free amplification (EFA) and label-free graphene oxide (GO)-based SYBR Green I fluorescence platform for detection of miRNA. MiRNA-21 was used as an example to demonstrate the feasibility of the method. Results show that the sensitivity of miRNA-21 is 1 pM, and the linearity range is from 1 pM to 1 nM. The method can specifically discriminate miRNA-21 from miRNA-210 and miRNA-214. Three tumor cell lines of A549, HepG2 and MCF7 were detected by the method. The sensitivities of them were 10^2 cells, 10^3 cells and 10^3 cells respectively. Clinical tumor samples were also tested by this method, and 29 of 40 samples gave out positive signals. The method holds great promise in miRNA detection due to its convenience, rapidness, inexpensive and specificity.

1. Introduction

MicroRNAs (miRNAs) are a kind of small single-stranded RNAs with the length of 18–25 nt (Ambros, 2004; Cissell et al., 2008; Zhou et al., 2010; Zhu et al., 2013; Cho, 2007; Cissell et al., 2007; Sedaghat-Hamedani et al., 2013). They involve in many important life activities and they have higher expression levels in many kinds of cancers (Hwang and Mendell, 2006; Yue and Tigyi, 2006; Khan et al., 2011; Liao et al., 2014). Therefore, quantification of miRNAs is great import in clinical diagnosis of cancer. However, due to miRNAs' character of small size, sequences similarity and vulnerable degradability, quantitative detection of miRNAs is always difficult. Thus, an evolutionary detection mode for microRNA is desired.

In recent years, with the emergences of novel amplification strategies and signal giving-out patterns, the microRNA detection method achieves considerable progresses. Traditional methods frequently use enzyme-based amplification reactions to realize high sensitivity and specificity. The usage of enzymes will need particular reaction time and conditions to maintain the enzyme's activities. Recently, an enzyme-free amplification (EFA) method has been developed (Yin et al., 2008; Li et al., 2011; Chen et al., 2013; Jiang et al., 2013; Liao et al., 2014). It overcomes the disadvantages of enzymatic amplification. Up to now, EFA has been combined with fluorescence and electrochemiluminescence (ECL) detection platform. However, these methods all used luminophore labeled probes as signal probes. (Yin et al., 2008; Li et al., 2011; Chen et al., 2013; Jiang et al., 2013; Liao et al., 2014).

Simultaneously, consideration of reducing test cost and time and label-free strategies without using luminophore labeled probes have gained tremendous interests in the biosensor fields. However, label-free strategies are confronted with the challenge of probe construction and signal giving-out pattern. Fortunately, with the development of nanotechnology, more label-free design strategies are selectable. With the emergence of nanomaterials, graphene oxide (GO) has attracted great interest in the biosensor fields due to its brilliant physical and chemical properties (Hea et al., 1998; Lerf et al., 1998; Guo et al., 2011; Lau et al., 2010; Pisula et al., 2011; Liu et al., 2012; Lv et al., 2012; Xu et al., 2012). It exhibits different affinity toward ssDNA versus double-stranded (ds) DNA and high fluorescence quenching ability (Lu et al., 2009,

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2010, 2009; Dong et al., 2010; He et al., 2010; Li et al., 2010; Lu et al., 2010; Wang et al., 2010; Wen et al., 2010; Lv et al., 2012). Based on this principle, a series of graphene-based biomolecular sensing technologies have been developed (Lu et al., 2009; Dong et al., 2010; He et al., 2010; Li et al., 2010; Lu et al., 2010; Wang et al., 2010; Wen et al., 2010; Zhou et al., 2013; Zhu et al., 2014).

In this study, we combine the isothermal and simplified EFA with the label-free GO-based SYBR Green I fluorescence switch platform for the detection of miRNA. To the best of our knowledge, it is the first reported isothermal enzyme-free amplification combined with label-free fluorescence platform for the detection of miRNA.

2. Experimental section

2.1. Materials and reagents

Forty clinical tumor tissue samples are gift from The First Affiliated Hospital of Jinan University, Guangzhou, China. All oligonucleotides and probes were synthesized and purified by Invitrogen. Graphene oxide (1 mg/mL) was purchased from Nanjing XFNANO Materials Tech Co., Ltd. SYBR Green I was purchased from Invitrogen. Total-RNA extraction kit, RNAase inhibitor and DEPC-treated water were purchased from Takara Biotechnology (Dalian) CO., LTD. The regents related to electrophoresis and phosphate buffered saline buffer (20 ×) were products of Shanghai Sangon Biotechnology Co., Ltd. Other chemicals were purchased from Sigma-Aldrich.

2.2. Preparation of probes

For the detection of target miRNA-21, two hairpin probes (H1 and H2) were constructed according to the theory of EFA and the sequence of miRNA-21. The sequences of the two hairpin probes are listed in Table 1. Before EFA, both H1 and H2 were denaturized at 95 °C for 5 min and then gradient cooling down (5 °C/min) to the room temperature, to form a complete hairpin structures. The probes are then stored at 4 °C until use (Liao et al., 2014).

2.3. RNA extraction from different cell lines and clinical tumor samples

Normal human liver cell line (LO2), human lung cancer cell line (A549), human hepatocellular liver carcinoma cell line (HepG2), and human breast adenocarcinoma cell line (MCF-7) are processed with cell counting. Forty clinical tumor tissue samples were pretreated by liquid nitrogen and grinded into paste. Then, RNAs were extracted from the cell lines and the clinical tumor samples using the total-RNA extraction kit.

2.4. Enzyme-free amplification

The EFA system contains 50 nM H1 and 50 nM H2, 0.8X PBS buffer, and 1 U/μL RNAase inhibitor in a total volume of 100 μL of DEPC-treated water. The mixture was incubated at 38 °C for 1 h for amplification.

2.5. Polyacrylamide gel electrophoresis

To test the feasibility of our constructed EFA assay, the EFA products were analyzed by 10% native polyacrylamide gel electrophoresis. Ten microlitre of samples were loaded and run at 120 V for 45 min at room temperature, and then dyed by SYBR Green I and photographed by Bio-Rad digital imaging system.

2.6. Detection of EFA products by GO-based SYBR Green I fluorescence platform

SYBR Green I and GO were added to 80 μl of enzyme-free amplification product in 1 × SSC buffer in turn, to make a final concentration of 1X and 8 μg/mL. After incubation for GO adsorption at 37 °C for 10 min, the fluorescence of SYBR Green I was measured by Perkin-Elmer LS55 luminescence spectrometer (USA).

3. Results and discussion

3.1. Experimental approach

This study aims to construct an EFA and GO-based SYBR Green I fluorescent switch platform for detection of miRNA. The overall steps of this method are shown in Fig. 1. Briefly, two hairpin probes (H1 and H2) are carefully designed according to the principle of EFA and the sequence of target miRNA. H1 includes six regions named as 1*, 2*, 3*, 4, 3, 2. The fragments 1*, 2*, 3* are recognition domains, which are entirely complementary with the target sequence. Fragment 1* provides an outshoot to open up H1, and fragments 2* and 3* are essential domains for construction of H1 hairpin structure. H2 contains five regions named as 3*, 4*, 3, 2, 4. The fragments 3*, 4*, 3, 2 are complementary to H1. Fragment 3* provides an outshoot to open up H2 (Liao et al., 2014). When no target exists, H1 and H2 keep their ssDNA hairpin structures. On the contrary, when target miRNA exists, hybridization of target with H1 will lead to open up the stem of H1, thus exposing the concealed sequence of H1's stem which is complementary to H2. The H1–target complex is then incubated with H2, and the hybridization of H2 to H1 will set the target miRNA free from the H1–H2 complex. The free target miRNA can be reused in the next EFA cycle. Therefore, one target miRNA can trigger many cycles of EFA and produce many copies of dsDNA H1–H2 complex. The amplification products are then incubated with GO and SYBR Green I for 10 min. GO exhibits different affinity toward ssDNA versus dsDNA and high fluorescence quenching ability. Therefore, when no target

Table 1
Sequences of microRNAs and hairpin probes.

Note	Sequences (5′→3′)					
microRNA-21	UAG CUU AUC AGA CUG AUG UUG A					
microRNA-210	CUG UGC GUG UGA CAG CGG CUG A					
microRNA-214	ACA GCA GGC ACA GAC AGG CAG U					
H1	1*	2*	3*	4	3	2
	TCAACATC	AGTCTGA	TAAGCTA	CCATGTGTAGA	TAGCTTA	TCAGACT
H2	3*	4*	3	2	4	
	TAAGCTA	TCTACACATGG	TAGCTTA	TCAGACT	CCATGTGTAGA	