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Electrochemical determination of microRNA-21 based on graphene, LNA integrated molecular beacon, AuNPs and biotin multifunctional bio bar codes and enzymatic assay system

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ABSTRACT

MicroRNAs (miRNAs), a kind of small, endogenous, noncoding RNAs (~22 nucleotides), might play a crucial role in early cancer diagnose due to its abnormal expression in many solid tumors. As a result, label-free and PCR-amplification-free assay for miRNAs is of great significance. In this work, a highly sensitive biosensor for sequence specific miRNA-21 detection without miRNA-21 labeling and enrichment was constructed based on the substrate electrode of dendritic gold nanostructure (DenAu) and graphene nanosheets modified glassy carbon electrode. Sulfydryl functionalized locked nucleic acid (LNA) integrated hairpin molecule beacon (MB) probe was used as miRNA-21 capture probe. After hybridized with miRNA-21 and reported DNA loading in gold nanoparticles (AuNPs) and biotin multi-functionalized bio bar codes, streptavidin–HRP was brought to the electrode through the specific interaction with biotin to catalyze the chemical oxidation of hydroquinone by H_2O_2 to form benzoquinone. The electrochemical reduction signal of benzoquinone was utilized to monitor the miRNA-21 hybridization event. The effect of experimental variables on the amperometric response was investigated and optimized. Based on the specific confirmation of probe and signal amplification, the biosensor showed excellent selectivity and high sensitivity with low detection limit of 0.06 pM. Successful attempts are made in miRNA-21 expression analysis of human hepatocarcinoma BEL-7402 cells and normal human hepatic L02 cells.

1. Introduction

MicroRNAs (miRNAs) are small, endogenous, noncoding RNAs (~22 nucleotides) found in diverse organisms, which play crucial roles in cell proliferation, differentiation and apoptosis. Up to date, 1426 miRNAs have been identified in the human genome. Recent researches have shown that abnormal expression of miRNAs is associated with a variety of human cancers (Esquela-Kerscher and Slack, 2006). Among various miRNAs, miRNA-21 has been identified as the only miRNA over-expressed in 11 types of solid tumors, including stomach, prostate, head and neck, esophagus, glioblastoma, neuroblastoma, cholangiocarcinoma, breast, lung, colorectal, and pancreatic cancer (Lu et al., 2008; Medina and Slack, 2008). Therefore, miRNA-21 could be potential biomarkers for clinical diagnosis. In order to understand the functions of miRNA-21 in diseases diagnosis, there is an urgent need to develop reliable and ultrasensitive methods for miRNA-21 determination.

At the present time, miRNA-21 is detected with techniques such as Northern blot (Kim et al., 2010), in situ hybridization (Yamamichi et al., 2009), RT-PCR (Huang et al., 2009), microarrays (Yan et al., 2008) and bioluminescence assay (Cissell et al., 2008). Although Northern blot and in situ hybridization are used as the standard methods, these detection approaches have low sensitivity and generally require many steps, resulting laborious time-consuming procedures that are difficult for routine miRNA analysis (Catuogno et al., 2011). RT-PCR can only be used to quantify miRNA precursors rather than the mature miRNAs expression, because the low detection selectivity and nonlinear target amplification might distort gene expression. Microarray needs fluorescent dyes labeled with miRNA target sequences to characterize the hybridization event, which might quench from the excitation light or from environment effects and require expensive instrument. Direct hybridization of miRNA samples onto the microarray requires a large amount of total RNA (Castoldi et al., 2008). Moreover, northern blot, RT-PCR and microarray need expensive commercial kits. Bioluminescence

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assay needs to label and enrich miRNAs. In addition, it requires trivial operation, expensive instruments and reagents.

To enhance the sensitivity, improve the selectivity, and make the detection limit and cost low, electrochemical biosensors based on signal amplification have been investigated to detect miRNAs due to their high sensitivity and selectivity, low detection limit, low cost, and ease of automatization (Drummond et al., 2003). For instances, Gao's group developed a series of sensitive electrochemical biosensors for monitoring miRNAs hybridization based on miRNAs labeled with $Os(dmpy)_2(IN)Cl^+$ (Gao and Yu, 2007b), $Ru(PD)_2Cl_2Os$ (Gao and Yu, 2007a), OsO_2 nanoparticles (Gao and Yang, 2006) and RuO_2 nanoparticles (Peng and Gao, 2011; Peng et al., 2010), respectively. Though the detection limit can be achieved to 800, 200, 80, 3 and 2 fM, respectively, this kind of monitor methods need to enrich and label miRNAs, which will increase the operation complicacy because of the low abundance of miRNAs in real samples. For decreasing influence of tagging and enriching miRNAs, and achieving the direct detection of miRNA in real sample, Kelly's group utilized electrostatic interaction between miRNA and $Ru(NH_3)_6^{3+}$ and the electrocatalytic effect between $Ru(NH_3)_6^{2+/3+}$ and $Fe(CN)_6^{4-/3-}$, a detection limit as low as 10 aM was achieved with a limited dynamic range of 10^2 (Yang et al., 2009). Lusi et al. (2009) exploited a simple and fast label-free electrochemical genosensor for miRNAs detection based on guanine oxidation consequent to the hybrid formation between the microRNA and its inosine substitute capture probe. Pöhlmann and Sprinzl (2010) developed a rapid, selective, and sensitive gap hybridization assay for detection of mature miRNAs without label based on four components DNA/RNA hybridization and electrochemical detection using esterase 2-oligodeoxynucleotide conjugates with the detection limit of 2 aM. All of these detection techniques showed high sensitivity, efficiency and selectivity, indicating that electrochemical biosensors can be alternative to other detection techniques.

In this work, we described a sensitive and selective miRNA biosensor based on graphene and dendritic gold nanostructure (DenAu) modified glassy carbon electrode (GCE), LNA integrated molecular beacon probe, multifunctional encoded DNA–AuNPs–LNA bio bar codes and HRP catalysis signal amplification, in which, the molecular beacon was not labeled with fluorescent and quencher groups, only a —SH was labeled at its 5′-end for assembly on the electrode surface. DNA–Au bio bar codes have become increasingly incorporated bioassays with its effective amplification based on AuNPs functionalized with a large number of oligonucleotide strands. Different from previous reports, the AuNPs were labeled with two oligonucleotide strands. One is LNA integrated DNA, which was complementary to the 3′-end of molecular beacon probe. The other is biotin functionalized DNA, not complementary to probe. The length of the probe (41 bases) was longer than the target miRNA (22 bases). The 5′-end of the molecular (5′–3′, No. 6–27 bases) was complementary to miRNA and its 3′-end (5′–3′, No. 28–41 bases) was complementary to LNA integrated DNA in DNA–AuNPs–LNA bio bar codes. After hybridization with target miRNA, the stem-loop structure of molecular beacon was unfolded and its 3′-end was forced far away from the electrode surface to hybridize with LNA integrated DNA. Then, with specific interaction between biotin and streptavidin, the streptavidin functionlized horseradish peroxidase (streptavidin–HRP) can be immobilized on the electrode surface to catalyze the oxidation reaction of hydroquinone by H_2O_2 to form benzoquinone and enhance the electrochemical reduction signal of benzoquinone (Chen et al., 2011; Laczka et al., 2011; Zhang et al., 2011). The detection strategy was shown in Scheme 1. Taking advantage of tri-amplification effects of the DenAu/graphene/GCE, multifunctional encoded AuNP and HRP, the biosensor showed high determination sensitivity for miRNA with detection limit of 0.06 pM (S/N = 3).

2. Experimental

2.1. Materials and apparatus

All oligonucleotides were synthesized and HPLC-purified by TaKaRa Biotechnology Co., Ltd. (Dalian, China), and their sequences are shown as follows, LNA integrated molecular beacon probe: 5′-SH—$(CH_2)_6$-GGC CGT CAA **C**AT CAG TC**T** GAT AAG C**T**A AAC ATG ATA CGG CC-3′; target miRNA-21 (S1), 5′-UAG CUU AUC AGA CUG AUG UUG A-3′; single-based mismatch miRNA, 5′-UAG CUU AUC ***G***GA CUG AUG UUG A-3′; three-based mismatch miRNA, 5′-U***U***G CUU AUC ***G***GA CUG AU***C*** UUG A-3′; non-complementary miRNA, 5′-GUA AGG CAU CUG ACC GAA GGC A-3′; LNA integrated reported DNA (S2), 5′-SH—$(CH_2)_6$-GG**C** **C**GT A**T**C ATG T**T**-3′; signal DNA (S3), 5′-SH—$(CH_2)_6$-GCG GAA CAC TCA AG-3′-biotin (nucleotide mismatches were indicated as italic and bold letters, locked nucleic acid bases were indicated as underlined and bold letters, the complementary section for probe to target miRNA-21 was indicated as bold letters with shade.). Synthetic DNA and miRNA sequences were dissolved in DEPC treated Milli-Q water with autoclaved sterilization and kept frozen. Graphene was synthesized according to previous report (Li and Wu, 2009). AuNPs were synthesized according to previous reports by chemical reduction using sodium citrate as reductant (Liu and Lu, 2006). Streptavidin labeled horseradish peroxidase (streptavidin–HRP), polyethylene glycol 3350 (PEG-3350) and ethidium bromide (EB) were purchased from Sigma (USA). Streptavidin–HRP was diluted 100 times with 10 mM phosphate buffer solution (PBS, pH 7.0). TRIzol reagent was from Invitrogen (USA). Diethypyrocarbonate (DEPC) was from Solarbio (China). Mercaptopropanoic acid (MPA), tri(2-carboxyethyl) phosphine hydrochloride (TCEP), tris(hydroxymethyl)aminomethane (Tris), disodium ethylenediaminetetraacetic acid (EDTA), sodium citrate, chloroauric acid and polyethylene glycol 3350 (PEG-3350) were purchased from Aladdin (Shanghai, China). All reagents were analytically pure grade.

The buffer solutions employed in this study are as follows. Probe immobilization buffer: 10 mM Tris–HCl, 1.0 mM EDTA, 1.0 M NaCl, and 1.0 mM TCEP (pH 7.0), miRNA hybridization buffer: 1 × SSC, DNA hybridization buffer: 10 mM Tris–HCl, 1.0 mM EDTA, and 1.0 M NaCl (pH 7.0), electrochemistry determination buffer, 0.1 M PBS (pH 7.0). All reagents were analytically pure grade. All of the solution and redistilled deionized water used were treated with DEPC and autoclaved to protect from RNase degradation.

Electrochemical experiments were performed with CHI660C electrochemical workstation (USA) with a conventional three-electrode cell. A bare GCE or modified GCE was used as working electrode. A saturated calomel electrode (SCE) and a platinum wire were used as the reference electrode and auxiliary electrode, respectively. Transmission electron microscopy (TEM) image was taken with JEOL-1200EX instrument (Japan). Atomic force microscopy (AFM) measurement was performed on Digital Instruments MultiMode (USA). RNA concentration and quality were assessed spectrophotometrically at 260 and 280 nm using NanoVue TM equipment (GE-Healthcare, Buckinghamshire, UK). All the measurements were carried out at room temperature (25 ± 0.5 °C).

2.2. Preparation of bio bar coded gold nanoparticles

The preparation of multifunctional encoded DNA–AuNPs bio bar codes was performed according to previous report with minor revision (Hu et al., 2008). In brief, the mixture of 5.0×10^{-10} mol of LNA integrated reported DNA and 2.0×10^{-9} mol of signal DNA was activated with acetate buffer (pH 5.2) and 1.5 μL of 10 mM TCEP for 1 h, then added to 1 mL of freshly prepared gold nanoparticles synthesized by chemical reduction, and shaken gently for 24 h. Then, the DNA–AuNPs conjugates were aged in salts (0.1 M NaCl, 10 mM