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DNA nanostructure-based ultrasensitive electrochemical microRNA biosensor

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ABSTRACT

MicroRNAs (miRNAs) are key regulators of a wide range of cellular processes, and have been identified as promising cancer biomarkers due to their stable presence in serum. As an surface-based electrochemical biosensors which offer great opportunities for low-cost, point-of-care tests (POCTs) of disease-associated miRNAs. Nevertheless, the sensitivity of miRNA sensors is often limited by mass transport and the surface crowding effect at the water-electrode interface. Here, we present a protocol as well as guidelines for ultrasensitive detection of miRNA with DNA nanostructure-based electrochemical miRNA biosensor. By employing the three-dimensional DNA nanostructure-based interfacial engineering approach, we can directly detect as few as attomolar (<1000 copies) miRNAs with high single-base discrimination ability. Since this ultrasensitive electrochemical miRNA sensor (EMRS) is highly reproducible and essentially free of prior target labeling and PCR amplification, it can conveniently and reliably analyze miRNA expression levels in clinical samples from esophageal squamous cell carcinoma (ESCC) patients.

1. Introduction

There have been increasing interests in the development of rapid, sensitive, and cost-effective DNA sensing devices for sequence-specific detection of clinically, environmentally, or security-relevant nucleic acid targets. MicroRNAs (miRNAs) as promising cancer biomarkers can regulate a wide range of cellular processes at the post-transcriptional level. Since the discovery of miRNAs in Caenorhabditis elegans in 1993 [1], there has been tremendous interest in studying their pivotal roles in basic biological processes and their expression levels in various types human cancers [2].While there has been urgent need for quantitative miRNA detection both in fundamental biological studies and for diagnostic purposes, it largely remains a technical challenge due to the low abundance, short length and sequence similarity of miRNAs [3]. In recent years, many novel techniques and methods have been reported for miRNA detection, including nanopore sensors [4], fluorescent sensors [5], colorimetric sensors [6], microarray based sensor [7] and the widely used Northern blotting [8] and quantitative polymerase chain reaction (qPCR) [9,10]. Although there have been advancements with each of these methods, none of the existing methods satisfy the high standards for point-of-care testing (POCT) of miRNAs, i.e. a label-free and amplification-free method that possesses sufficiently high sensitivity and selectivity to detect very minute miRNA from serum samples, specificity to identify 1–2 mismatches within the miRNA family, and low cost and portability for applications in small clinics and/or at home.

Electrochemical sensors are well recognized to be promising point-of-care testing (POCT) device due to the ready availability of inexpensive and small-size electrochemical detectors (e.g. electrochemistry-based ubiquitous glucose meters) [11,12]. However, the sensitivity of electrochemical DNA sensors is often limited by the accessibility of target DNA/RNA molecules to probes attached to the heterogeneous electrode surface due to the reduced mass transport and the presence of surface crowding effect (in contrast to probe-target recognition in homogeneous solution) [13–16]. Hence, the sensitivity of electrochemical sensors for miRNAs (pM–fM) usually does not support direct detection of low-abundance miRNAs without prior amplification with PCR.

With the rapid emergence of DNA nanotechnology, it has been able to 'bottom-up' construct exquisite DNA nanostructures with excellent controllability and high precision arising from unmatched self-recognition properties of DNA molecules [17,18]. Our previous studies have demonstrated that a three-dimensional (3D) DNA tetrahedron-structured probe (TSP) modified with sulfur at three vertices can be rapidly and firmly adsorbed at gold surfaces [19], resulting in a nanoengineered interface which can improve the ability of biomolecular sensing [19,20]. The nanoengineered interface could provide a convenient solution to spatial

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control and enhanced accessibility of probes on the surface without relying on advanced micro/nano-fabrication technologies.

Here we exploit this DNA nanostructure-based interfacial engineering strategy and the base stacking-based strategy for short-sequence miRNAs detection. We employed the base-stacking strategy to overcome the problem of "sandwich-type" based electrochemical miRNAs (short length and low melting temperature) assays and developed an a label-free and PCR-free electrochemical miRNA sensor (EMRS) for ultrasensitive detection of attomolar miRNAs with extraordinarily high sequence specificity.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Oligonucleotides (Table 1; Invitrogen Inc.)
Synthesized mature miRNAs (Table 2; Invitrogen Inc.)
Total RNAs from tissue in three human organs (liver, prostate and lung; Ambion)
Total RNA samples from esophageal squamous cell carcinoma (ESCC) patients (provided by Zhongshan Hospital of Shanghai)
TMB substrate (TMB = 3, 3′, 5, 5′ tetramethylbenzidine; K-blue low activity substrate; Neogen)
Horseradish peroxidase-conjugated avidin (avidin-HRP; Roche Diagnostics)
Streptavidin-poly-HRP80 (poly-HRP80; Fitzgerald Industries International Inc.)
poly-HRP diluent (Fitzgerald Industries International Inc.)
Ethylene glycol-terminated thiol (HS-$(CH_2)_{11}$-EG_2-OH, OEG; Prochimia)
Diethyl pyrocarbonate, >97% (DEPC; Sigma) **!CAUTION** DEPC is hazardous of skin contact, eye contact and inhalation, which is irritant to skin, eyes and respiratory.
Tris (2-carboxyethyl) phosphine hydrochloride (TCEP; Sigma)
Tris-(hydroxymethyl)aminomethane (Tris base; Cxbio Biotechnology Ltd.)
Sulfuric acid, >98% (H_2SO_4; Sinopharm Chemical Reagent Co., Ltd.) **!CAUTION** H_2SO_4 is dangerously corrosive and can cause severe burns.
Ethylenediaminetetraacetic acid (EDTA; Sigma)
Sodium phosphate monobasic, >99% (NaH_2PO4; Sinopharm Chemical Reagent Co., Ltd.)
Sodium phosphate dibasic dodecahydrate, >99% ($Na_2HPO_4 \cdot 12H_2O$; Sinopharm Chemical Reagent Co., Ltd.)
Potassium phosphate dibasic, >99% (KH_2PO_4; Sinopharm Chemical Reagent Co., Ltd.)
Sodium chloride, >99.5% (NaCl; Sinopharm Chemical Reagent Co., Ltd.)
Potassium chloride, >99.8% (KCl; Sinopharm Chemical Reagent Co., Ltd.)
Magnesium chloride, >99.5% ($MgCl_2$; Sinopharm Chemical Reagent Co., Ltd.)
Ethanol (Sinopharm Chemical Reagent Co., Ltd.)
Milli-Q water (18 MΩ cm; Millipore)

Table 2
Sequences of mature human miRNAs.

miRBase ID	Sequence (5′–3′)	Accession#
hsa-mirR-21	UAGCUUAUCAGACUGAUGUUGA	MIMAT0000076
hsa-miR-31	AGGCAAGAUGCUGGCAUAGCU	MIMAT0000089
hsa-let-7a	UGAGGUAGUAGGUUGUAUAGUU	MIMAT0000062
hsa-let-7b	UGAGGUAGUAGGUUGUGUGGUU	MIMAT0000063
hsa-let-7c	UGAGGUAGUAGGUUGUAUGGUU	MIMAT0000064
hsa-let-7d	AGAGGUAGUAGGUUGCAUAGUU	MIMAT0000065
hsa-let-7e	UGAGGUAGGAGGUUGUAUAGUU	MIMAT0000066
hsa-let-7f	UGAGGUAGUAGAUUGUAUAGUU	MIMAT0000067
hsa-let-7g	UGAGGUAGUAGUUUGUACAGUU	MIMAT0000414
hsa-let-7i	UGAGGUAGUAGUUUGUGCUGUU	MIMAT0000415
hsa-miR-98	UGAGGUAGUAAGUUGUAUUGUU	MIMAT0000096

2.1.2. Equipments

CHI630b electrochemical workstation (CH Instruments Inc.)
CHI101 2-mm-diameter Gold working electrode (CH Instruments Inc.)
CHI115 platinum wire counter-electrode (CH Instruments Inc.)
CHI111 Ag/AgCl reference electrode (CH Instruments Inc.)
CHI220 cell stand with Φ25 mm × 40 mm glass cell (CH Instruments Inc.)
Alpha alumina powder, 0.3 mm (CH Instruments Inc.)
Gamma alumina powder, 0.05 mm (CH Instruments Inc.)
Microcloth (CH Instruments Inc.)
Himac CF16RX versatile compact centrifuge, with a model T15A34 rotor (Hitachi Koki Co., Ltd.)
U-3010 spectrophotometer (Hitachi High-Technologies Corporation)
KQ218 ultrasonic cleaning (Kunshan Ultrasonic Instruments Co., Ltd.)
Milli-Q synthesis A10 (Millipore Inc.)
Thermomixer comfort (Eppendorf Inc.)
IKA lab dancer (IKA Inc.)
HD-850 Horizontal air flow clean bench (Shanghai sujing Industrial Co., Ltd.)
PCR Peltier thermal cycler PTC-200 (MJ. Research Inc.)

2.1.3. Reagent setup

All solutions were prepared with RNase-free water. The RNase-free water was prepared with Milli-Q water (18 MΩ cm resistivity) treated with 0.1% DEPC.

Table 1
Synthetic oligonucleotide probes.

Probe name	Sequence (5′–3′)
tetra-miR-21	ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTAAAAAAAAAAATCAACATCAG
tetra-let-7d	ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTAAAAAAAAAAAAACTATGCAA
tetra-miR-31	CATCTTGCCTAAAAAAAAAAAACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTA
tetra-B	SH-C_6TATCACCAGGCAGTTGACAGTGTAGCAAGCTGTAATAGATGCGAGGGTCCAATAC
tetra-C	SH-C_6-TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGCGGCTCTTC
tetra-D	SH-C_6-TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGACCCTCGCAT
swRP-miR-21	TCTGATAAGCTA-BIOTIN′
swRP-let-7d	CCTACTACCTCT-BIOTIN′
swRP-miR-31	BIOTIN′-AGCTATGCCAG
SH-ss-miR-21	SH-C_6-TAAATAAATATCAACATCAG
miD-21	TAGCTTATCAGACTGATGTTGA