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Catalytic hairpin assembly-based electrochemical biosensor with tandem signal amplification for sensitive microRNA assay

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We demonstrate for the first time the construction of a low background electrochemical biosensor with tandem signal amplification for sensitive microRNA assay based on target-activated catalytic hairpin assembly (CHA) of heteroduplex-templated copper nanoparticles. This electrochemical biosensor exhibits high sensitivity, good specificity, single-base mismatch discrimination capability, excellent stability and reproducibility, and it can sensitively detect microRNA in cancer cells.

MicroRNAs (miRNAs) are small noncoding RNAs and may function as the sequence-specific post-transcriptional regulators.[1] They convey genetic information from DNAs to proteins through specifying the amino acid sequence of the protein products of gene expression in ribosome, and their expression levels are associated with many human diseases including cancers.[2-4] The miRNAs have become promising biomarkers for early cancer diagnosis, and the sensitive biosensors for accurate quantification of miRNA expression levels in cancer cells are highly desirable.[5-8] Recently, a series of nanoclusters/nanoparticles-based biosensors have been developed for miRNA assay.[9-13] Especially, the introduction of oligonucleotide-templated nanoclusters/nanoparticles greatly improves the performance of biosensors.[9,10] Since DNAs have high affinity towards metal cations, they may function as the DNA templates to selectively form the metal nanoparticles. The resultant nanoparticles can be further used as the nucleation sites for the deposition of metal nanoparticles (e.g., silvers,[11-13] golds,[14] palladium[15] and platinum nanoparticles[16]). Yang report the in situ synthesis of silver nanoclusters (AgNCs) using the cytosine-rich loop DNA templates for label-free electrochemical detection of miRNA (miRNA-199a).[12] Zhang demonstrate that the AgNCs/DNA nucleated by a cytosine-rich sequence can integrate with strand-displacement amplification (SDA) for miRNA assay.[13] Nevertheless, the synthesis of oligonucleotide-templated silver nanoclusters requires special sequence DNA.[12,13]

DNA-templated copper nanoparticles (CuNPs) exhibit distinct advantages of non-toxicity, low cost and ease of preparation,[9,17,18] and may in situ synthesized as the environment-friendly nano-dyes for signal transduction.[19] However, they usually involve more than one type of the carefully designed DNAs (e.g., the use of thymine-rich ssDNAs for the formation of fluorescent CuNPs),[19,20] and suffer from fluorescence decay. Mokhir reported the use of dsDNA as the template for the synthesis of CuNPs with high fluorescence at low-concentration $CuSO_4$, and they found that the ssDNA template cannot generate CuNPs.[21] The resultant CuNPs can accumulate in the major groove of dsDNA instead of ssDNA. Dai used the DNA–RNA heteroduplexes as the templates to synthesize the copper nanocluster (CuNC) for the construction of an electrocatalysis-assisted electrochemical biosensor,[4] but this kind of biosensor relies on the H_2O_2-mediated peroxidase catalytic reaction with the characteristics of instability, easy decomposition of externally H_2O_2, and poor sensitivity.[22-24]

In this research, we demonstrate for the first time the construction of a low background electrochemical biosensor with the capability of tandem signal amplification for sensitive miRNA assay based on miRNA-activated catalytic hairpin assembly (CHA) of heteroduplex-templated copper nanoparticles. The target miRNA-21 can trigger the strand displacement assembly of two hairpin substrates, resulting in the cyclic reuse of target miRNA-21. The alkaline phosphatase (ALP) catalyzes the hydrolysis of a substrate ascorbic acid 2-phosphatase (AAP), producing a reductive product ascorbic acid (AA) whose catalysis reduction may result in the in-situ formation of CuNPs with dsDNA as the template for the generation of an amplified electrochemical signal. Notably, there is no strict requirement for the sequence of dsDNA template, facilitating the design of different biosensors for the detection of various biomarkers.

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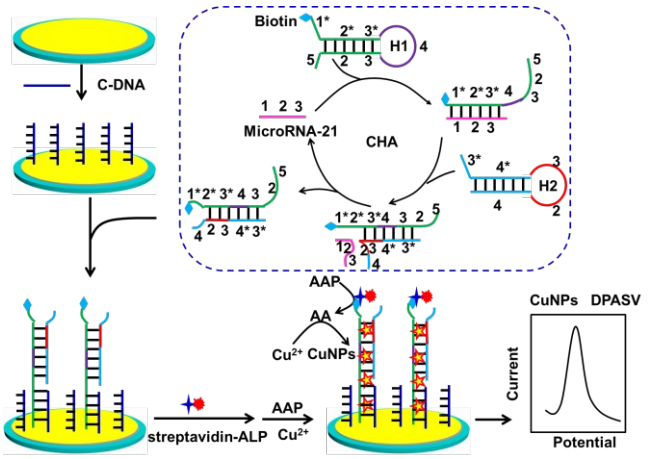

Scheme 1. Schematic illustration of electrochemical biosensor for miRNA assay based on miRNA-activated catalytic hairpin assembly (CHA) (with hairpin 1 (H1) and hairpin 2 (H2)) of heteroduplex-templated copper nanoparticles.

As proof of concept, we used miRNA-21 as the target miRNA. Scheme 1 shows the construction of an electrochemical biosensor for miRNA assay based on miRNA-activated catalytic hairpin assembly (CHA) of heteroduplex-templated copper nanoparticles. We designed two stem-loop DNA molecular beacons (H1 and H2) for CHA reaction (see ESI,† Table S1), with H1 being labeled by biotin at 5′-end. The thiolated capture DNAs (C-DNAs) are self-assembled on the surface of a gold electrode via Au-S bond. In absence of target miRNA-21, the probe H1 remains intact and cannot automatically hybridize with H2. When target miRNA-21 is present, it hybridizes with H1 to form the H1-miRNA-21 intermediate, initiating the dynamic assembly of H2 and the simultaneous release of miRNA-21. The released miRNA-21 can trigger another cyclic hybridization, eventually leading to the formation of abundant H1-H2 double-stranded complexes. Subsequently, the C-DNAs immobilized on the gold electrode can hybridize with the H1-H2 complexes through domains 2 and 5. Notably, the hybridization of H1 with H2 enables the displacement of target miRNA-21 by H2, and the released miRNA-21 may initiate cyclic CHA. The biotinylated H1 can bind streptavidin-alkaline phosphatases (streptavidin-ALPs) through biotin-streptavidin interaction, and the resultant ALP can catalyze the hydrolysis of ascorbic acid 2-phosphatase (AAP) substrate, generating a reductive product ascorbic acid (AA) which can mediate the in situ synthesis of CuNPs. Double-stranded DNA (dsDNA) obtained from the hybridization of C-DNA with CHA product on the gold electrode surface may function as the template for in situ synthesis of CuNPs. The resultant CuNPs on the gold electrode surface can be dissolved by HNO_3 to form Cu^0 which is subsequently oxidized to Cu^{2+}. The produced Cu^{2+} was electrodeposited on a glassy carbon electrode, generating an enhanced electrochemical signal as a result of differential pulse anodic stripping voltammetry (DPASV) of Cu^{2+}.

We used polyacrylamide gel electrophoresis (PAGE) to verify this assay. As shown in Fig. 1A, the band of single-stranded C-DNA with the smallest molecular weight is observed with the fastest moving rate (Fig. 1A, lane 1). In addition, both H1 and H2 are stable in the absence of miRNA-21 (Fig. 1A, lane 2). Upon the addition of miRNA-21, the distinct band of amplification product H1-H2 complexes is observed with a slower moving rate (Fig. 1A, lane 3), indicating the successful CHA reaction. The subsequent hybridization of C-DNAs with the H1-H2 duplexes, resulting in the formation of the C-DNA-H1-H2 complexes with the largest molecular weight and the slowest moving rate (Fig. 1A, lane 4), accompanied by the decrease of the H1-H2 complexes band. We further measured the anodic stripping voltammetric (ASV) signal to verify this assay (Fig. 1B). The electrochemical signal in response to target miRNA-21 (Fig. 1B, curve b) is 26.8-fold higher than that in the presence of the C-DNA-modified Au electrode without target miRNA-21 (Fig. 1B, curve a), suggesting that this electrochemical biosensor can be used to detect target miRNA-21.

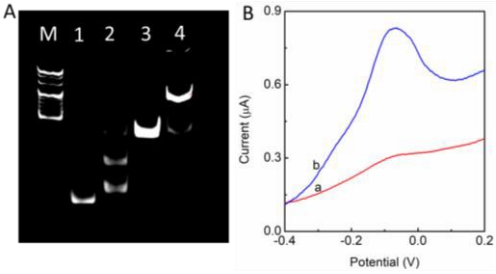

Fig. 1 (A) Polyacrylamide gel electrophoresis of reaction products. Lane M: DNA marker; Lane 1: C-DNA; Lane 2: H1 + H2; Lane 3: H1 + H2 + miRNA-21; Lane 4: C-DNA + H1 + H2 + miRNA-21.The 1 μM H1, 1 μM H2 and 1 μM miRNA-21 were used in the experiments. (B) Differential pulse voltammograms of glassy carbon electrode (GCE) after electrodeposition of Cu^{2+} dissolved from CuNPs formed on different DNA-modified gold electrodes: treatment of C-DNA with the catalytic hairpin assembly in the absence (a) and presence (b) of target miRNA-21.

To experimentally verify the construction of the proposed electrochemical biosensor, we measured the cyclic voltammetry (CV) (see ESI,† Fig. S1A) and the electrochemical impedance spectra (EIS) (see ESI,† Fig. S1B) of the DNA-modified electrodes in different construction steps. We further quantified the density of DNA on the electrode surface (see ESI,† Fig. S2) using chronocoulometry. The surface density of C-DNA on the gold electrode surface is estimated to be 7.005×10^{12} molecules cm^{-2} (see ESI,† Fig. S2), suggesting that the electrode surface can be further modify by the CHA products.

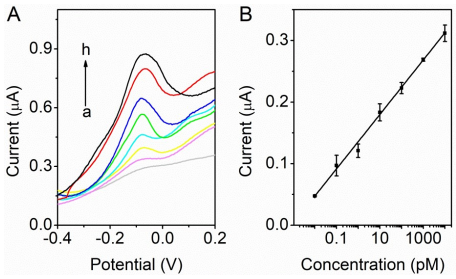

Fig. 2 (A) DPV curves in response to target miRNA-21 from a to h: 0, 0.01, 0.1, 1, 10, 100, 1000, 10000 pM. (B) Relationship between the electrochemical response and the logarithm of miRNA-21 concentration in the range from 10 fM to 10 nM. Error bars are standard derivation obtained from three independent experiments.

Under the optimally experimental conditions (see ESI,† Fig. S3), we measured the DPV curves in response to different concentrations of target miRNA-21 from 10 fM to 10 nM (Fig. 2A). As shown in Fig. 2B, the DPV peak current enhances with the logarithm of miRNA-21 concentration in the range of 0.01 – 10000 pM. The obtained equation is I = - 0.67 + 0.044 $\log_{10} C$ (R^2 = 0.999), where I (μA) is the DPV peak current and C is the concentration of miRNA-21 (pM). The limit of detection (LOD) is calculated to be 1.1 fM by evaluating the average value of the control plus 3 times standard deviation. The sensitivity of this electrochemical biosensor is much higher than those of the reported methods based on CHA (see ESI,†Table S2), with 3.6-fold higher than that of fluorescent assay,[25-28] 3.9-fold higher than that of electrochemical assay,[29,30] 8.4-fold higher than that of colorimetric assay.[31] The high sensitivity of this electrochemical biosensor can be attributed to the involvement of tandem signal amplification: (1) the target miRNA-21 can open the stem of H1 by hybridization reaction, exposing the concealed sequence of H1 stem, initiating CHA for the achievement of an amplified signal; (2) the dsDNA can function as the template for the synthesis of CuNPs under mild condition, but ssDNA cannot be used for the synthesis of CuNPs, resulting in an extremely low background.

We further investigated the specificity of this electrochemical biosensor by measuring the complementary target miRNA-21, the single-base mismatch miRNA-21 (M-miRNA-21), miRNA-210, and miRNA-214, respectively. As shown in Fig. 3, the DPV peak currents in response to 10 nM miRNA-210 and 10 nM miRNA-214 are similar to that of the control group without miRNA-21. In contrast, the DPV peak current in response to 10 nM target miRNA-21 is 18-, 11.4-, 12.4- and 3.0-fold higher than that of the control group without miRNA-21, 10 nM miRNA-210, 10 nM miRNA-214 and 10 nM M-miR-21, respectively. Notably, the DPV peak current in response to even extrembly low-concentration target miRNA-21 (10 pM) is 2.2-fold higher than that in response to M-miR-21 (10 pM), suggesting that this electrochemical biosensor possesses the single-base mismatch discrimination capability. In addition, the DPV signal exhibits little fluctuation with a relative standard deviation (RDS) of 3% and 4.8% after 7 days and 15 days of storage, respectively (see ESI,† Fig. S4A), indicating the good stability of the proposed electrochemical biosensor. The RSD for intra-assay (2.7%) and inter-assay (3.8%) exhibits no significant difference under identical conditions (see ESI,† Fig. S4B), indicating good reproducibility of the proposed electrochemical biosensor.

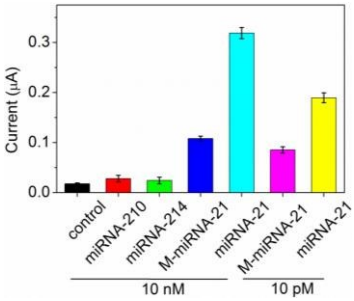

Fig. 3 Measurement of DPV peak current in response to the control group without any sample (black column), 10 nM miRNA-210 (red column), 10 nM miRNA-214 (green column), 10 nM M-miRNA-21 (blue column), 10 nM miRNA-21 (wathet column), 10 pM M-miRNA-21 (purple column), and 10 pM M-miRNA-21 (yellow column), respectively. Error bars are standard derivation obtained from three independent experiments.

To demonstrate the capability of the proposed electrochemical biosensor for real sample analysis, we measured the level of miRNA-21 in total RNA of human breast cancer cell line (MCF-7 cells). The total RNA sample was diluted to 1 μg μL^{-1} with diethylpyrocarbonate (DEPC)-treated water, and 5 μL of total RNA sample was subjected to measurement. As shown in Fig. 4A, the electrochemical signal in response to 5 μg of total RNA can be well distinguished from that in response to the control without miRNA-21. According to the calibration curve in Fig. 2B, the amount of miRNA-21 in 5 μg of total RNA sample was estimated to be 1.8 fmol, consistent with the previous report.[32,33] When 9 fmol of synthesized miRNA-21 was spiked into 5 μg of total RNA, the absolute amount of miRNA-21 in the spiked sample was quantified to be 11.3 fmol with a recovery rate of 104.5% (RSD = 2.9%, n = 5), consistent with the previous report,[34] demonstrating that the proposed electrochemical biosensor can be applied to detect miRNA in complex biological samples. As shown in Fig. 4B, the peak current exhibits a linear correlation with the logarithm of MCF-7 cell number in the range from 5 to 10000 cells. The regression equation is I = 0.030 – 0.073 $\log_{10} N$ (R^2 = 0.993), where I is the peak current (μA) and N is the number of MCF-7 cells. The LOD of the proposed electrochemical biosensor is calculated to be 3 cells by evaluating the average value of the control plus 3 times standard deviation. The sensitivity of the proposed electrochemical biosensor is comparable to that of the reported electrochemical biosensor based on the integration of catalytic hairpin assembly with rolling circle application (3 cells).[35] This result clearly demonstrates that the proposed electrochemical biosensor can be used to quantitatively detect cellular miRNA-21, holding great potential for further applications in clinical diagnosis.

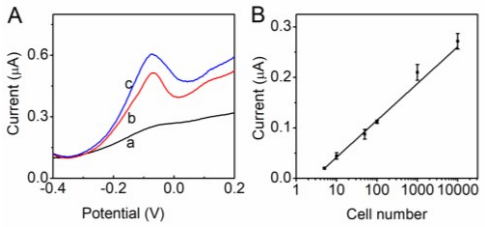

Fig. 4 (A) Measurement of DPV curves in response to the control without miRNA-21 (a), total RNA (b), total RNA + synthesized miRNA-21 (c). (B) Linear relationship between the peak current and the logarithm of MCF-7 cell number. Error bars are standard derivation obtained from three independent experiments.

In summary, we have demonstrated for the first time the construction of a low background electrochemical biosensor with the capability of tandem signal amplification for sensitive miRNA assay based on miRNA-activated catalytic hairpin assembly of heteroduplex-templated copper nanoparticles. This electrochemical biosensor can achieve tandem signal amplification with following distinct characteristics: (1) the

dsDNA can be used as the template for the synthesis of CuNPs under mild conditions, but ssDNA cannot be used for the synthesis of CuNPs, resulting in an extremely low background; (2) the use of dsDNA as the template without special sequence facilitates the design of different biosensors for the detection of various biomarkers; (3) miRNA-activated catalytic hairpin assembly (CHA) of heteroduplex-templated copper nanoparticles greatly amplifies the electrochemical signal; (4) ALP catalyzes the hydrolysis of AAP to produce AA for the synthesis of CuNPs without the involvemnet of external AA, greatly simplifying the experimental procedures and decreasing the background signal. This electrochemical biosensor takes advantage of the dynamic DNA nanotechnology and oligonucleotide-templated copper nanoparticles, exhibiting high sensitivity, good specificity, single-base mismatch discrimination capability, excellent stability and reproducibility, with potential applications in early diagnosis of cancers. Importantly, by simply changing the corresponding sequence of hairpin DNA, this electrochemical biosensor can be extended to detect a variety of miRNAs and DNAs.

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Conflicts of interest

There are no conflicts to declare.

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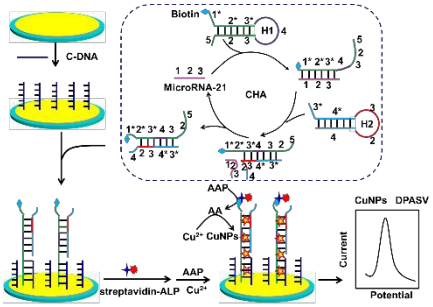

We develop an catalytic hairpin assembly-based electrochemical biosensor with tandem signal amplification for sensitive microRNA assay.