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Highly sensitive and label-free electrochemical detection of microRNAs based on triple signal amplification of multifunctional gold nanoparticles, enzymes and redox-cycling reaction

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ABSTRACT

MicroRNAs (miRNAs) are believed to be important for cancer diagnosis and prognosis, serving as reliable molecular biomarkers. In this work, we presented a label-free and highly sensitive electrochemical genosensor for miRNAs detection with the triple signal amplification of gold nanoparticles (AuNPs), alkaline phosphatase (ALP) and p-aminophenol (p-AP) redox cycling. The label-free strategy is based on the difference in the structures of RNA and DNA. Specifically, miRNAs were first captured by the pre-immobilized DNA probes on a gold electrode. Next, the cis-diol group of ribose sugar at the end of the miRNAs chain allowed 3-aminophenylboronic acid (APBA)/biotin-modified multifunctional AuNPs (denoted as APBA–biotin–AuNPs) to be attached through the formation of a boronate ester covalent bond, which facilitated the capture of streptavidin-conjugated alkaline phosphatase (SA–ALP) via the biotin–streptavidin interaction. After the addition of the 4-aminophenylphosphate (p-APP) substrate, the enzymatic conversion from p-APP to p-AP occurred. The resulting p-AP could be cycled by a chemical reducing reagent after its electro-oxidization on the electrode (known as p-AP redox cycling), thus enabling an increase in the anodic current. As a result, the current increased linearly with the miRNAs concentration over a range of 10 fM–5 pM, and a detection limit of 3 fM was achieved. We believe that this work will be valuable for the design of new types of label-free and sensitive electrochemical biosensors.

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1. Introduction

MicroRNAs (miRNAs) are 18- to 25-nucleotide-long non-coding RNA molecules that play roles in developmental and cell biology, including developmental regulation, stress responses, cell differentiation, cardiogenesis and epigenetic inheritance. Recently, the aberrant expression of miRNAs has been correlated with cancer (prostate, breast, colon, lung, etc.) and other diseases (diabetes, heart diseases, etc.), making miRNAs clinically important biomarkers and drug discovery targets (Dong et al., 2013; Tran et al., 2013). The methods currently used for miRNAs detection, such as Northern blotting, microarrays and polymerase chain reaction (PCR), are usually time-consuming, less sensitivity and/or require fluorescent- or radio-labeling and complicated instrumentation (Nelson et al., 2004; Schmittgen et al., 2004; Streit et al., 2009). Therefore, it is critical to develop robust detection methods for miRNAs with high sensitivity, selectivity and simplicity (Cissell et al., 2007; Zhang et al., 2009).

In recent years, electrochemical genosensors have held great promise as devices suitable for point-of-care diagnostics and multiplexed platforms for fast, simple and inexpensive nucleic acid analysis (Paleček and Bartošík, 2012). A typical electrochemical genosensor is made of a solid electrode with an immobilized short single-stranded nucleotide probe for hybridization with the complementary sequence (Lu et al., 2008a; Tosar et al., 2010; Xie et al., 2004). Once hybridization occurs, there must be a way to translate the hybridization event into a measurable signal. One of the limiting factors for the development of electrochemical miRNAs sensors is sensitivity, as the miRNAs content is at the attomolar to femtomolar level in biological samples (Cissell et al., 2007). Recently, great efforts have been made to develop sensitive electrochemical genosensors for the detection of low-abundance miRNAs (Dong et al., 2012; Gao et al., 2013; Gao and Yu, 2007; Gao and Yang, 2006; Hong et al., 2013; Kilic et al., 2012, 2013; Lusi et al., 2009; Pöhlmann and Sprinzl, 2010; Peng and Gao, 2011; Ren et al., 2013; Shen et al., 2013; Wang et al., 2013; Yin et al., 2012b). Signal amplification is the most popular strategy for the development of ultrasensitive assay methods (Duan et al., 2013). Normally, signal amplification can be achieved using a nanoparticle or enzyme catalytic reaction (single amplification)

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or a nanoparticle/enzyme conjugate catalytic reaction (double amplification). These methods often require the labeling of nanoparticles with complementary probes for the recognition of miRNAs or the use of labeled DNA/RNA for a competitive binding assay. The practical applications of these methods are therefore limited due to their time-consuming and costly preparation, the instability of the modified nanoparticle, and their low sensitivity for real sample assays.

A more recent approach for signal amplification is to employ multiple signal amplification, such as a catalytic reaction plus a redox-cycling reaction (Yang, 2012). In redox cycling, electrochemically oxidized (or reduced) species are reduced (or oxidized) electrochemically, enzymatically, or chemically. The regenerated electroactive species are electrochemically re-oxidized (or re-reduced), resulting in high electrochemical signals. For example, Das et al. (2006) and Tang et al. (2011) suggested that antibody-coated gold nanoparticles (AuNPs) can improve the sensitivity of electrochemical immunoassays using p-aminophenol (p-AP) redox cycling. Yang's group reported a series of chemical amplification approaches using an enzymatic reaction and electrochemical–chemical redox cycling by different reducing reagents on indium-tin oxide (ITO) electrodes (Akanda et al., 2011, 2012, 2013; Das et al., 2007). These electrochemical immunoassays allow low-concentration proteins (femtomolar or below) to be detected readily.

The hydroxyl group at the 2′ position of the ribose sugar provides the RNA molecule with a cis-diol group at the 3′-terminal. This property enables miRNAs to be distinguished from DNA. For example, based on the difference in the structures of RNA and DNA, Gao's group developed an electrochemical biosensor for miRNAs detection using OsO_2 nanoparticles as tags for miRNAs labeling (Gao and Yang, 2006). Paleček's group demonstrated that the 3′-end of miRNAs can be selectively modified with Os(VI)2,2′-bipyridine (Trefulka et al., 2010). Phenylboronic acids can form boronate ester covalent bonds with diol-containing biomolecules (e.g., dopamine, sugars, nucleosides, antibodies and glycoproteins) (Abad et al., 2002; Frasconi et al., 2010; Ho et al., 2010; Lin et al., 2009, 2011; Liu et al., 2013b; Song et al., 2012; Xia et al., 2013a). The capture, separation and immobilization of nucleotides and RNA with boronic-acid-functionalized materials have also been achieved by several groups (Deore and Freund, 2005; Li et al., 2011; Pham et al., 2010; Potter et al., 2006; Rahman and Elaissari, 2012; Zayats et al., 2002). Recently, our group reported a label-free strategy for miRNAs detection based on the dual amplification of 4-mercaptophenylboronic acid (MBA)-capped and dopamine (DA)-modified AuNPs (Xia et al., 2013b). As a result, a detection limit of 50 fM was achieved. In the present work, we developed a highly sensitive and label-free electrochemical method for miRNAs detection based on the triple signal amplification of multifunctional AuNPs, alkaline phosphatase (ALP) and p-AP redox-cycling reaction. Specifically, miRNAs captured by the DNA probes on a gold electrode were derivatized with 3-aminophenylboronic acid/biotin-modified multifunctional AuNPs through the formation of boronate ester covalent bonds. The derivation allowed streptavidin-conjugated alkaline phosphatase (SA–ALP) to be attached for the production of electrochemically active p-AP. The produced p-AP could be cycled by a chemical reducing reagent after its electrooxidization on the electrode, increasing the anodic current.

2. Experimental

2.1. Chemicals and reagents

Tris(carboxyethyl)phosphine (TCEP), 6-mercapto-1-hexanol (MCH), 3-aminophenylboronic acid (APBA) hydrochloride, streptavidin-conjugated alkaline phosphatase (SA–ALP), bovine serum albumin (BSA), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), KH_2PO_4, K_2HPO_4, trisodium citrate and tris-(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) were purchased from Sigma-Aldrich. 4-Aminophenylphosphate (p-APP) was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Diethylpyrocarbonate, adenosine-5′-monophosphate (AMP), cytosine-5′-monophosphate (CMP) and a thiolated single-stranded DNA (ss-DNA) probe (5′-TCAACATCAGTCTGATAAGCTA-$(CH_2)_6$-SH-3′) were purchased from Sangon Biotech. Co., Ltd. (Shanghai, China). The target miRNA-21 and the mismatch sequences were obtained from GenePharma Co., Ltd. (Shanghai, China) with the following sequences: 5′-UAGCUUAUCAGACUGAUGUUGA-3′ (miRNA-21), 5′-UAGCUUAUCGGACUGAUGUUGA-3′ (single-based mismatch), 5′-UUGCUUAUCGGACUGAUCUUGA-3′ (three-based mismatch) and 5′-GUAAGGCAUCUGACCGAAGGCA-3′ (non-complementary). The peptides (CALNN and CALNNGK(biotin)G) were synthesized and purified by ChinaPeptides Co., Ltd. (Shanghai, China). All other reagents were purchased from Beijing Chemicals, Ltd. (Beijing, China). The DNA solutions were prepared using TE buffer solution (10 mM Tris–HCl, 1 mM EDTA, pH 7.4), and kept at −18 °C. The miRNAs stock solutions were prepared daily with diethylpyrocarbonate-treated water in an RNase-free environment. The hybridization solution was prepared with TNE buffer (TE+0.1 M NaCl). The buffer (EB) for the electrochemical experiment was 10 mM Tris, 1 mM $MgCl_2$ and 50 mM Na_2SO_4 (pH 7.4).

2.2. Formation of boronate esters between the nucleotide and APBA

The formation of boronate esters between nucleotide and APBA was confirmed by mass spectroscopy (LCT Premier XE). APBA and the nucleotide (AMP or CMP) were dissolved in deionized water at 100 and 20 μM, respectively. The pH of the APBA/nucleotide mixed solution was then adjusted to 8.0 with 1 M NaOH and the mass spectra were collected in negative ion mode.

2.3. Synthesis and characterization of multifunctional AuNPs

All glassware used in the following procedure was cleaned in a bath of freshly prepared 1:3 HNO_3–HCl, rinsed thoroughly with water and dried in air prior to use. The peptide-functionalized AuNPs (denoted as biotin–AuNPs) were prepared by ligand-exchange reaction between CALNN and CALNNGK(biotin)G peptides and citrate-stabilized AuNPs, as in previous reports (Lévy et al., 2004; Wang et al., 2005). Briefly, trisodium citrate (5 mL, 38.8 mM) was rapidly added to a boiling solution of $HAuCl_4$ (50 mL, 1 mM), and the solution was boiled continually for an additional 30 min to yield a red-wine-colored solution. The particle concentration of the synthesized AuNPs suspension was determined to be 10.2 nM based on a molar absorptivity of $2.7 \times 10^8\ M^{-1}\ cm^{-1}$ at 520 nm. The ligand-exchange reaction was then performed at room temperature by mixing 5 mL of the as-prepared AuNPs suspension with CALNN and CALNNGK(biotin)G at room temperature for 2 h. The concentration ratio of CALNN to CALNNGK(biotin)G was 9:1, as in the previous study (Lévy et al., 2004). The immobilization ability of AuNPs for the peptides was studied by measuring the free peptides in solution with mass spectroscopy. The results indicated that CALNN and CALNNGK (biotin)G molecules at concentrations of 6.3 and 0.7 μM were almost completely absorbed by the 10.2 nM AuNPs suspension (see Fig. S1 in Supplementary material). Thus, the average number of CALNN and CALNNGK(biotin)G molecules per gold nanoparticle was estimated to be 618 and 69, respectively. The suspension was thoroughly rinsed with deionized water to remove the lower amounts of free peptides. The 3-aminophenylboronic acid (APBA)-modified biotin–AuNPs (denoted as APBA–biotin–AuNPs) were prepared by cross-linking APBA molecules onto the biotin–AuNPs surface via the EDC-mediated amine coupling reaction

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