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Simultaneous detection of multiple DNA targets based on encoding metal ions

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ABSTRACT

We present a novel strategy for simultaneous electrochemical detection of multiple DNA targets based on the use of different encoding metal ions as tags. The principle of this scheme is that metal ions bound to metallothionein (MT) molecules can be released down after hybridization with DNA targets and then be detected by stripping voltammetry. The novel detection probes, ssDNA/MT conjugates, covered with different metal ions were synthesized for the first time, then three encoding metal ions (Zn^{2+}, Cd^{2+}, and Pb^{2+}) were used to differentiate the signals of three virus DNA due to their well-defined anodic stripping peaks at −1.13 V (Zn), −0.78 V (Cd), and −0.52 V (Pb) at BiFE, respectively. The anodic peak currents increased linearly with the concentrations of DNA targets in the range from 0.1 nM to 10 nM with a detection limit of 33 pM. In addition, the one-base mismatched target was effectively discriminated from the complementary target. The described results demonstrated that this method possesses high sensitivity and selectivity for multi-target DNA assay and has great potential in applications for detection of even more targets in biological assays, particularly immunoassays.

1. Introduction

The detection of DNA sequences is of central importance because of its broad applications in clinical diagnosis, genetics therapy, and a variety of biomedical studies (Klepárník and Bocek, 2007; Riccardi et al., 2007). Recently, the development of highly sensitive and selective DNA sensors for simultaneous analysis of multiple DNA targets is a field of ever increasing interest. Many detection methodologies rely on optical readouts, such as chemiluminescence (Freeman et al., 2011), fluorescence (Cui et al., 2008; He et al., 2010; Su et al., 2012), surface-enhanced Raman scattering (SERS) (MacAskill et al., 2009; Kang et al., 2010; Maiti et al., 2012) and so on. For example, Itamar Willner's group demonstrated that the three different sized CdSe/ZnS quantum dots (QDs) can be used for chemiluminescence resonance energy transfer (CRET) assays of multiple DNA targets (Freeman et al., 2011). Daxiang Cui' team reported on multiplexed DNA analysis based on three CdSe QDs with different emission wavelengths (Cui et al., 2008), while Karen Faulds et al. described a SERRS assay employing three dye-labeled probes (MacAskill et al., 2009). Although these strategies could achieve a higher sensitivity for single-target assay, it is difficult to identify the single target in a mixture because of the broad emission spectrum or the complicated background signals (MacAskill et al., 2009).

On the other hand, electrochemical detection of DNA, as an effective method, has attracted considerable interest due to its simplicity, low-cost and high sensitivity (Liu et al., 2008; Wei et al., 2008; Loaiza et al., 2010). Within recent years, several inventive designs for multi-target detection have appeared based on the detection probes labeled with different electroactive tags (Wang et al., 2003; Hansen et al., 2006; Zhang et al., 2009). QDs have been also applied for multiplexed electrochemical DNA sensors reported by Gothelf and Wang (Wang et al., 2003; Hansen et al., 2006). In their works, three different QDs (ZnS, CdS, and PbS) were used as tags to differentiate the signals for simultaneous analysis of three DNA targets. However, the preparation of QDs is often a time-consuming work and requires harsh conditions (Wang et al., 2003), and it is also difficult to control their sizes.

In order to develop a simple and sensitive method for multiplexed DNA assays, we try to use metal ions as the tags to detect three different virus DNA segments simultaneously. But research found that many heavy (or transition) metal elements can damage DNA molecules and alter the fidelity of DNA synthesis (Świątek et al., 1987; Tajmir-Riahi et al., 1993; Burda et al., 1997). Therefore, labeling the DNA probes with metal ions directly is still a challenge. In this article, an approach was proposed based on metallothionein (MT) molecules as a bridge to combine ssDNA and metal ions. The novel detection probes, ssDNA/metallothionein (MT) conjugates, covered with different metal ions were synthesized for the first time, then after hybridization with DNA targets, metal ions bound to MT molecules can be released down in the acidic solution and be detected by stripping voltammetry. The new

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strategy can offer a remarkably low detection limit because of its effective 'built-in' deposition of the Bi^{3+} and target metal ions (Zn^{2+}, Cd^{2+}, and Pb^{2+}). On the other hand, the hybridization signal, to a certain extent, will be further enhanced since each MT could bind to more than one metal ion.

2. Materials and methods

2.1. Materials

The MT (> 99%) from rabbit liver was purchased from Botai Bio-tech (Dalian, China). Succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane 1-carboxylate (SMCC) came from Pierce. DL-Dithiothreitol (DTT) and 1-hexanethiol (MCH) were acquired from Aladdin (Shanghai, China). $BiCl_3$ was obtained from Shanghai No. 2 Chemicals Factory (Shanghai, China). $ZnSO_4 \cdot 7H_2O$, $CdCl_2 \cdot H_2O$, $Pb(NO_3)_2$, CH_3COOH, $CH_3COONa \cdot 3H_2O$, $HClO_4$, dimethyl sulfoxide (DMSO), ethylenediaminetetracetic acid (EDTA), $AgNO_3$, NaOH, Tris (hydroxymethyl) aminomethane (Tris), HCl, NaCl, NaH_2PO_4 and Na_2HPO_4 were purchased from Beijing Chemical Reagents Co. (Beijing, China). Other chemicals employed were all of analytical grade, and Milli-*Q* water was used in all experiments. All oligonucleotides were provided by Sangon (Shanghai, China). The capture probes were thiolated with a $-(CH_2)_3-$ spacer at the 3′ ends and the detection probes were modified with amino groups with a $-(CH_2)_6-$ spacer at the 5′ ends. Their base sequences were:

human enterovirus-related oligonucleotides:

capture probe (c_1): 5′-CAGACACTGTTGGTA-$(CH_2)_6$-SH-3′
detection probe (d_1): 5′-H_2N-$(CH_2)_6$-GAATAGCGTCAGAAT-3′
complementary target (t_1): 5′-TACCAACAGTGTCTGATTCT-GACGCTATTC-3′
one-base mismatched target (t_1'): 5′-TACCGACAGTGTCT-GATTCTGACGCTATTC-3′
four-base mismatched target (t_1''): 5′-TACCGGAACTGTCT-GATTCTGACGCTATTC-3′

human herpesvirus 3-related oligonucleotides:

capture probe (c_2): 5′-GCTAAAACACGCGGC-$(CH_2)_6$-SH-3′
detection probe (d_2): 5′-H_2N-$(CH_2)_6$-GTGACATCGCTATGT-3′
complementary target (t_2): 5′-GCCGCGTGTTTTAGCACATAGC-GATGTCAC-3′

foot-and-mouth disease virus-related oligonucleotides:

capture probe (c_3): 5′-TGCATCTGGTTAATG-$(CH_2)_6$-SH-3′
detection probe (d_3): 5′-H_2N-$(CH_2)_6$-GTTGACATGTCCTCC-3′
complementary target (t_3): 5′-CATTAACCAGATGCAGGAGGA-CATGTCAAC-3′

2.2. Metallation of apo-MT

The lyophilized MT was dissolved in 0.02 M Tris–HCl buffer at pH 8.6, and was treated with 100 molar equivalents of DTT for 1 h under nitrogen at room temperature. The pH was adjusted to 1.0 by the rapid addition of HCl (for preparation of Zn-MT and Cd-MT) or by $HClO_4$ (for preparation of Pb-MT). Then the MT was washed by 0.05 M HCl or 0.1 M $HClO_4$ several times to remove any bound metal ions by a Centricon-3 centrifugal concentrator. Then, apo-MT (metal-free MT) was converted to the fully metallated form based on a previously reported (slightly modified) procedure (Vašák, 1991). Apo-MT (0.2 mg/mL) solution was placed in a round-bottom flask and 8 molar equivalents of any metal ion (Zn^{2+}, Cd^{2+}, or Pb^{2+}) solution was added. The solution was rendered oxygen free by gently agitating under a stream of nitrogen for 20 min and kept under a positive pressure of nitrogen. The solution was then titrated with 0.5 M Tris base (oxygen free) to the desired pH of 7.4 (for Zn-MT), 8.6 (for Cd-MT) or 7.6 (for Pb-MT) respectively with fast stirring and incubated for 1 h at room temperature. The excess metal ions were removed by ultrafiltration. The metallation of apo-MT was assessed by UV absorption spectra and square wave anodic stripping voltammetry (SWASV) measurements.

2.3. Conjugation of MT to NH_2-DNA

SMCC, the heterobifunctional crosslinker, was first dissolved in DMSO, and then diluted with water immediately prior to use to a final concentration of SMCC of 5%. To prevent the hydrolytic degradation of the NHS ester, the conjugation was usually performed at pH 7.3. Phosphate-buffered saline (PBS = 0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.4), the amine- and sulfhydryl-free buffer, was used as conjugation buffer. A 40-fold molar of crosslinker over the amount of MT was used to react simultaneously with MT and excess NH_2-DNA for 1 h at room temperature. The unreacted SMCC and NH_2-DNA were removed from the reaction product by ultrafiltration. The conjugation was assessed by UV absorption spectra and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Hybridization procedures

The 5 μL of TE (10 mM Tris–HCl and 1 mM EDTA) solution containing 2 μM capture probe was casted onto a gold substrate. To avoid water evaporation, the substrate was held up in a humidified Styrofoam chamber. Upon immobilization overnight, the substrate was washed with water and soaked in an aqueous solution containing 0.1 mM MCH solution for 2 h. After the surface was thoroughly rinsed with water, the substrate covered with a capture probe/MCH mixed SAM was further immersed in a sealed small centrifuge tube containing 200 μL of the detection probes and targets. Then the hybridization steps took place in a water bath at 60 °C for 5 min and allowed to cool down slowly. After hybridization, release of the metal ions from MT molecules was happened in 100 μL of 1 M HCl for 10 min. The acidic solution (containing metal ions) was transferred into a 0.1 M acetate buffer solution (pH 4.5) containing a certain amount of Bi^{3+} to perform SWASV measurements.

2.5. UV absorption spectra

The forming of Zn-MT was determined spectrophoto-metrically in 20 mM Tris/HCl at pH 7.4 and the conjugation of ssDNA/MT was also determined by UV absorption spectra in 0.1 M PBS, 0.15 M NaCl, pH 7.3. UV absorption spectra were recorded on a UV-1102 UV–vis spectrophotometer (Shanghai, China) in a 1 mm quartz cuvette at room temperature. The wavelength range of 220–300 nm was scanned continuously. All spectra were baseline-corrected.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoresis was performed at the SE 400 vertical unit (GE Healthcare). The concentration of the polyacrylamide gel was 18%. The electrophoresis started with an initial voltage of 30 V and