Biosensors and Bioelectronics 42 (2013) 51–55

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Ultrasensitive detection of melamine based on a DNA-labeled immunosensor

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ARTICLE INFO

Article history:
Received 27 August 2012
Received in revised form
26 October 2012
Accepted 26 October 2012
Available online 5 November 2012

Keywords:
Melamine detection
DNA-labeled immunosensor
Food safety

ABSTRACT

A DNA-labeled immunosensor for melamine (MEL) detection is presented which combined the exponential amplification and quantitative effect of Real-Time quantitative PCR (RT-qPCR) with the simplicity and specificity of competitive antigen–antibody reaction. An excellent linear relationship between cycle threshold (Ct) and MEL concentration in the range of 0.001–10 pg g^{-1} was obtained with a limit of detection of 0.3 fg g^{-1}. Compared with other methods, the sensitivity of this DNA-labeled immunosensor showed a 1000-fold improvement, and was below the strictest safety limit of 1 ppm. Furthermore, the specificity was excellent and the recovery in liquid milk samples spiked with MEL was satisfactory. With the advantages of high sensitivity and a low limit of detection (LOD), this sensor is a powerful and promising tool for the detection of other small molecules besides MEL.

1. Introduction

Melamine is a basic heterocyclic triazine organic compound extensively used in the manufacture of resins, plastics and glues, but is banned in food products and additives. Because of its high nitrogen content, it was illegally added to milk and pet food to increase the apparent crude protein levels measured by the Kjeldahl method (Chan et al., 2008). It is known that the high intake of MEL can contribute to long-term kidney failure and liver problems (Dobson et al., 2008; Hau et al., 2009; Nilubol et al., 2009). Although it is unclear whether MEL is hazardous to humans during chronic exposure to minute traces, there is still an urgent need to develop a novel method to lower the detection limit to meet future demand.

Many techniques have been developed to detect MEL (Sun et al., 2010), including traditional immunoassays such as the enzyme-linked immunosorbent assay (ELISA) (Choi et al., 2010; Lei et al., 2011; Liu et al., 2010; Yin et al., 2010) and gold immunochromatographic strip (Li et al., 2011; Sun et al. 2012), as well as instrumental based methods such as gas/liquid chromatography (GC/LC; Beltran-Martinavarro et al., 2012; Tittlemier et al., 2009), gas chromatography/mass spectrometry (GC/MS; Squadrone et al., 2010; Zhu et al., 2009), liquid chromatography–tandem mass spectrometry (LC–MS/MS; Fang et al., 2012), surface-enhanced Raman spectrometry (Wen et al., 2012), and surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS; Hsieh et al., 2012). In addition, colorimetric determination (Ai et al., 2009; Kuang et al., 2011; Ma et al., 2011; Zhang et al., 2012) and fluorimetric methods (Gao et al., 2012; Han et al., 2012; Wang et al., 2012) based on nanomaterials, and electrochemical sensors (Zhu et al., 2010) have also been developed. In the traditional immunoassays, many washing steps and optimization of working conditions are required to obtain high sensitivity. High instrument costs and complicated sample pretreatment are necessary in the instrumental methods. Professional technology is required for the synthesis of nanomaterials, which restricts the widespread implementation of this technique. Therefore, the development of methods for the detection of MEL with simple operation and high sensitivity is challenging.

PCR is widely used for DNA amplification and provides a precise and sensitive method for the determination of specific DNA in samples. Alternative methods for the detection of PCR products vary, and include gel electrophoresis and RT-qPCR (Arya et al., 2005; Branford et al., 2004; Ladero et al., 2012; Lee et al., 2009). Gel electrophoresis is a semi-quantitative technique to detect DNA content using a gel image analysis system. During RT-qPCR, the amount of DNA increases exponentially and the fluorescent signal produced is detected by the dye inserted into the dsDNA or the probe complementary to the template. The differences in initial DNA concentration are reflected in the Ct values, which can be used as a quantitative indicator. Because of the amplification effect and quantitative measurement by RT-qPCR, this has significant advantages with respect to handling time, LOD and reproducibility of the assay (Adler et al., 2003; Csordas et al., 2010; Jiang et al., 2012; Kim et al., 2011; Tian et al., 2012). A relationship between Ct value and target concentration has also been used for detection with a significant sensitivity enhancement, but the detection targets were mostly big molecules and the sandwich method was used (Babu and Muriana, 2011; Morin et al., 2011).

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http://dx.doi.org/10.1016/j.bios.2012.10.089

In this paper, we describe the design and construction of a new type of DNA-labeled immunosensor through which a target binding event can be reported by amplified RT-qPCR signals. This study only requires a single antibody against the target antigen immobilized in PCR tubes, and the detection effects were shown to be significantly better than traditional immunoassays using at least two types of antibodies, the capture antibody and detector antibody for the sandwich technique or the first and second antibody for the competitive method. The DNA sequence was selected randomly only if it had high proliferating ability, and was coupled to the antibody by a chemical coupling agent and amplified to quantify the content of antigen indirectly. At the same time, the whole procedure required fewer washing steps and was completed in only one PCR tube. In addition, the super signal amplification of PCR established the foundation for low LOD. These advantages make the technique simple resulting in its potential use for the ultrasensitive detection of MEL.

2. Materials and methods

2.1. Materials and reagents

Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) was purchased from Sigma-Aldrich, USA. Water used during the procedure was obtained from a Millipore Milli-Q purification system (Biocel). The coated antigen (MEL-OVA) and antibody against MEL were prepared in our lab. The SsoFast EvaGreen Supermix was from Rare Biotechnology Co. (Nanjing, China). The random probe sequence containing 5′-terminal sulfhydryl groups and the corresponding upstream and downstream primers were synthesized by Shengon Biotechnology Co. Ltd. (Shanghai, China). Their sequences are as follows:

Template: 5′-SH-GGGAAAATGCAAGAAGAAGTCATTAGTCCTA-GACAACGTTACTATAACGTGAATGTAATGAACCTACAAGACCTTCCA-GATTTTTCGGC-3′Upstream primer: 5′-GGGAAAATGCAAGAAGA-AGTCAT-3′Downstream primer: 5′-GCCGAAAAATCTGGAAGGTC-3′.

2.2. Preparation and purification of antibody–DNA conjugates

The monoclonal antibody against MEL was prepared before with an IC_{50} value of 6 μg L^{-1} in our lab (Sun et al., 2012). The DNA–antibody conjugates were synthesized according to the method reported by Boozer (Boozer et al., 2004). The antibody at 3 mg mL^{-1} reacted with Sulfo-SMCC and was dissolved in PBS (100 mM PBS, pH 7.4, 150 mM NaCl) at a ratio of 1:10. After incubating for 30 min at room temperature, the salt ions and the excessive reagent were removed by ultrafiltration (3000 MW cutoff membrane; Millipore). The PBE buffer (100 mM PBS, 5 mM EDTA) was changed to dissolve the intercepted molecule. An identical amount of ssDNA template and antibody was added, incubated for 30 min at room temperature, and the compound was obtained followed by ultrafiltration (10,000 MW cutoff membrane; Millipore) to eliminate unreacted DNA. Ultrafiltration was carried out twice to ensure the removal of unbound substance. The amount of DNA in the conjugate was determined based on the A260/280 ratios.

2.3. RT-qPCR assay for MEL detection

To improve the adsorbability of PCR tubes, each tube was treated with 50 μL 0.8% glutaraldehyde solution for 5 h at 37 °C. The tubes were subsequently washed with ultrapure water three times, coated with 50 μL MEL-OVA for 2 h at 37 °C and then washed with PBST (10 mM PBS, pH 7.2, 0.05% Tween-20) and block for 2 h at 37 °C with blocking buffer (10 mM PBS, pH 7.2, 0.4% gelatin). After that, 25 μL MEL standard sample of 10-fold serial dilutions and 25 μL DNA-labeled antibody specific to MEL were added to each tube at the same time and incubated for 30 min at 37 °C. All tubes were then washed five times with PBST to remove the unbound DNA-labeled antibody.

PCR mixture at a total volume of 50 μL was added to the washed tubes, and RT-qPCR was carried out directly in PCR tubes using the CFX-96 Real-Time PCR system. The PCR cycling parameters were an initial denaturation for 30 s at 95 °C, followed by 39 cycles of denaturation for 5 s at 95 °C, and annealing for 30 s at 57 °C. Fluorescence measurements were taken after each annealing step. A melting curve was obtained from 65 °C to 95 °C to detect potential nonspecific products, and the signal was acquired at every 0.5 °C.

2.4. Specificity analysis

Cephalosporin, which can be detected in milk products, was added instead of the MEL standard sample to validate specificity. All other procedures were identical to those mentioned above. At the same time, MEL structural analogs cytosine, thymine, uracil, and alanine were used to further evaluate the selectivity of the proposed immunosensor.

2.5. Recovery in fluid milk samples

Negative fluid milk samples were centrifuged at 12,000 r/min for 20 min to remove impurities which could interfere with the detection process. The obtained supernatants were diluted appropriately, and the MEL standard sample at five concentrations: 0.001, 0.01, 0.1, 0.5, and 1 pg g^{-1}, were added.

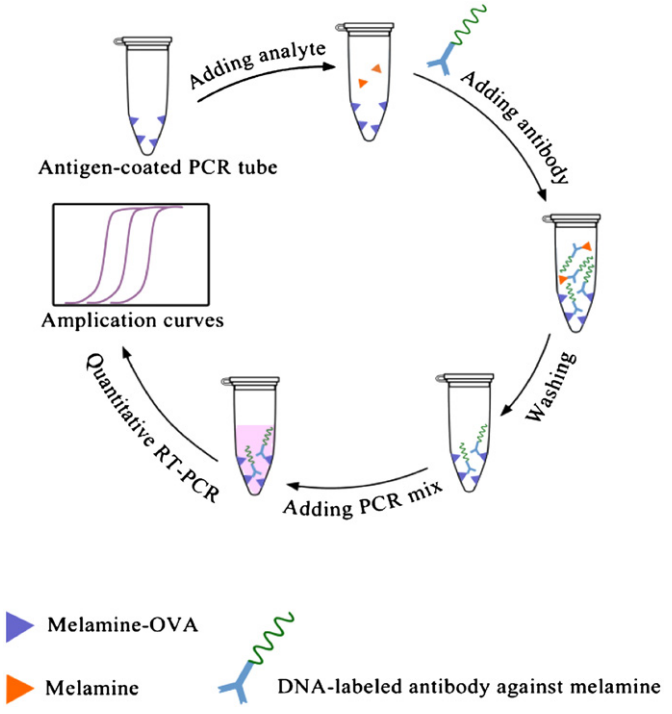

Fig. 1. Schematic diagram of DNA-labeled antibody based ultrasensitive MEL detection via Real-Time quantitative-PCR signal amplification.