Development of Immunochromatographic Strip Tests for Selective and Quantitative Detection of Melamine

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Abstract

An immunochromatographic strip test based on the complex reaction of antigen-antibody (melamine-antimelamine) was developed for quantitative detection of melamine. Gold nanoparticles (AuNP) were used to form AuNP-labeled antibody, which then acted as a biosensor. Melamine quantification was performed by the determination of AuNP using anodic stripping voltammetry technique with a boron-doped diamond as the working electrode. With sample volume of 100 µL and immunoreaction time of 7 min, the developed immunochromatographic strip test produced a linear calibration curve for melamine concentration range of 0–0.6 mg/L, with detection limit of 0.1 mg/L and RSD of ~5%. Furthermore, negative results were obtained for samples containing cyanuric acid and urea, indicating that the developed immunochromatographic strip test has potential for selective and quantitative detection of melamine.

Keywords: anodic stripping voltammetry, boron-doped diamond, gold nanoparticles, immunochromatographic strip test, melamine

1. Introduction

Melamine, 1,3,5-triazine-2,4,6-triamine (Figure 1) is a synthetic compound used as an industrial chemical in the production of plastics, amino resins, and flame retardants [1-3]. It was recently reported that melamine was deliberately added to milk and cheese [4] to provide a false indication of protein concentration, since the conventional method of protein detection (Kjeldahl method) determines nitrogen content only. Although melamine has low oral toxicity, some studies showed that high and continuous dietary exposure to melamine can cause renal stones and urinary bladder tumors [5-7]. Various analytical methods have been developed for the detection of melamine, such as gas chromatography, high-performance liquid chromatography, gas chromatography-mass spectrometry, liquid chromatography-tandem mass spectrometry, capillary electrophoresis, and enzyme-linked immunosorbent assay (ELISA) [8-12]. Although these methods offer high sensitivity and specificity, they nevertheless require expensive instruments and highly skilled analysis.

Recently, Li et al. [7] developed a strip test for rapid qualitative detection of melamine, based on immunochromatography. In another study, the same authors also

Figure 1. Structure of Melamine
also developed an electrochemical immunosensor device, based on a nanoparticle probe, for quantitative detection of proteins [13].

In this work, we developed an immunochromatographic strip test for melamine, combined with electrochemistry technique for detection of gold nanoparticles (AuNP). AuNP was used to label the melamine antibody (AuNP-antibody), which then functioned as a biosensor to recognize the antigen (melamine). The quantity of melamine was determined, based on the assumption that the quantity of AuNP involved in the system was representative of melamine concentration. Anodic stripping voltammetry (ASV) using boron-doped diamond (BDD) electrode as the working electrode was selected for the electrochemical detection of AuNP. This method was reported by Yamada et al. [14] to be highly suited to the detection of trace metals, providing high selectivity and stable current responses. The results of the present study show that the developed immunochromatographic strip test is promising for the selective and quantitative detection of melamine.

2. Experiment

Chemicals. Bovine serum albumin (BSA), Millipore glass-fiber filter, and nitrocellulose membrane (5 μm pore size) were purchased from Sigma Aldrich. Antibody of melamine was from Beacon. Melamine, trisodium citrate (Na₃C₆H₅O₇), trimethoxyborane (C₃H₅BO₃), and other chemicals were supplied by Wako. All chemicals were used without purification. Pure water was obtained from a simple water system (Direct-Q UV 3-Millipore). Pt wire and Ag wire were from Nilaco, and a peroxidase-antiperoxidase (PAP) pen super-liquid blocker was obtained from Cosmo Bio.

Preparation of gold nanoparticles (AuNP) and AuNP-labeled antibody. Colloidal AuNP was prepared by boiling 100 mL of 0.01% HAuCl₄ with constant stirring. Then, 2 mL of 1% trisodium citrate was added, followed by 15 min boiling. After the solution had cooled, distilled water was added to re-adjust the volume.

In order to prepare AuNP-labeled antibody, 10 mL of colloidal AuNP was prepared to pH 7 by the addition of 0.1 M potassium carbonate. Then, 500 μL of antibody was added. After an incubation time of 10 min at ambient temperature, BSA was added with constant gentle stirring, followed by a further 10 min incubation. The solution was centrifuged at 12,000 rpm for 15 min to obtain a red precipitate of AuNP-antibody, which was then dissolved with 2 mL of phosphate buffer solution (PBS) pH 7.4. A UV-Vis spectrometer (Jasco, V-570) and transmission electron microscope (TEM) (TECNAI) were used to characterize the AuNP and AuNP-labeled antibody.

Preparation of immunochromatographic strip test. The immunochromatographic strip tests consisted of several components, including a sample pad, a conjugate pad, a nitrocellulose membrane, a test zone, and an absorbent pad. All components were assembled on a piece of the plastic backing, as illustrated in Figure 2. The sample pad, the conjugated pad, and the absorbent pad were made of glass-fiber filters, whereas the immuno-chromatographic reaction path and the test zone were made of nitrocellulose membrane. The conjugate pad was prepared by carefully dropping 100 μL of AuNP-labeled antibody. After drying at room temperature, the procedure was repeated twice. Meanwhile, the test zone was prepared via the same method, using 100 μL of 5% antimelamine in 0.02 M PBS pH 7.4.

Preparation of the electrochemical device. The electrochemical device consisted of a piece of BDD as the working electrode, Pt wire (dia. 0.2 mm) as the supporting electrode, and an Ag/AgCl system as the reference electrode. BDD was prepared on a silicon wafer using microwave plasma-assisted chemical-vapor deposition (ASTeX Corp.) as described elsewhere [15]. A mixture of 50 mL acetone and 4 mL trimethoxyborane solution was used as carbon and boron sources, respectively. Raman spectra (Princeton Instrument, Acton sp2500) and SEM images (JEOL, JSM 5400) suggested that the prepared BDD was of high quality [16]. All electrodes were then assembled into a set of electrochemical cells on a plastic backing (inset of Figure 2) and placed under the test zone of the immunochromatographic strip test as the electrochemical measurements were performed.

Quantitative detection of melamine using immunochromatographic strip test. Melamine solution (volume 100 μL) of various concentrations was introduced on the sample pad of the strip test. The sample then moved...
through the nitrocellulose membrane and the conjugate pad, respectively, taking ~7 min to reach the test zone. Then, the test zone was underlined with two lines (distance between lines was ~10 mm) using PAP pen. A volume of 200 µL of 0.02 M HClO₄ was added into the test zone as supporting electrolyte, and after 2 min the measurements were performed using the ASV method.

3. Results and Discussion

Characterization of colloidal AuNP and AuNP-labeled antibody. UV-Vis spectrum (Figure 3) shows that the maximum absorbance of colloidal AuNP occurred at ~520 nm. However, when AuNP was conjugated to antibody of melamine and BSA, the wavelength shifted and the absorbance decreased. While the decrease in absorbance was probably due to increased distance between the AuNP particles, the bathochromic shift of the wavelength indicates increased AuNP diameter.

However, TEM images of AuNP (Figure 4a) and AuNP-labeled antibody (Figure 4b) show that when AuNP was conjugated to antibody and BSA, the average diameter of the particles appeared to be similar (~16 nm), whereas the distance between particles increased. It seems that AuNP was surrounded by much larger molecular size of antibody and BSA, which affects the absorbance wavelength. The surrounding BSA needs to be included in the labeling process to act as the active site of the antibody (paratope). Therefore, the antibody can interact with the antigen (melamine) in its epitope site [17]. BSA, which is an amino acid sequence, is expected to fill the ends of the “Y” antibody (antigen binding site) to enable the antibody to recognize and bind to melamine specifically [18].

Anodic Stripping Voltammetry of AuNP. Meanwhile, ASV was examined for AuNP detection. HClO₄ was used not only for the supporting electrolyte but also for the oxidizing agent of AuNP [14]. ASV involves two reaction steps, including reduction or pre-concentration of the analyte at the surface; and oxidation or stripping of the deposited analyte. Therefore, the change Au⁺ of AuNP to Au³⁺ ions is expected to provide suitable condition for ASV. A well-defined oxidation peak observed at the potential around +1.1 V seems to comply with the requirements for ASV (Figure 5a).

The oxidation reaction during the process is proposed below:
Step 1: Dissolution step using HClO₄
\[ \text{AuNP} \rightarrow \text{Au}^{3+} + 3e^- \] (1)
Step 2: Reduction or pre-concentration step
\[ \text{Au}^{3+} + 3e^- \rightarrow \text{Au}^0 \] (2)

Figure 3. UV-Vis Spectrum of AuNP in the Absence (Solid Line) and in the Presence of Antibody and BSA (Dotted Line)
Step 3: Oxidation or stripping step

\[ \text{Au}^{0} \rightarrow \text{Au}^{3+} + 3e^- \quad (3) \]

The oxidation peak current of the voltammogram was highest at the deposition potential of -0.4 V. Meanwhile, the oxidation peak continuously increased with deposition time from 60 s to 300 s. Therefore, deposition potential of -0.4 V and deposition time of 240 s were fixed for subsequent experiments. Furthermore, ASV for various concentrations of AuNP in HClO₄ shows linear dependence of the peak current at +1.1 V on the concentrations of AuNP (Figure 5b), suggesting that the peak can be utilized for AuNP detection.

**Electrochemical detection of melamine using immunochromatographic strip test.** When melamine standard solution is introduced to the sample pad, the sample moves due to the capillary nature of the nitrocellulose membrane. At the conjugated pad, melamine selectively interacts with the immobilized AuNP-labeled antibody to form the complexes of AuNP-labeled antibody-melamine. The immunoreaction is expected to complete through the nitrocellulose membrane toward the test zone. In the test zone, the complexes containing melamine are captured by capturing the antibody to form AuNP-labeled antibody-melamine-antibody sandwich complexes, whereas the complexes without melamine continuously move toward the absorbent pad. Since the complexes without melamine have much smaller size than the sandwich complexes of melamine, theoretically, it is possible to separate them through their velocity difference during movement within the nitrocellulose membrane. Movement of the red-colored AuNP from the sample pad to the test zone was observed to take ~7 min, which is estimated to be sufficient for the immunoreaction time [8].

Figure 6 shows voltammograms of various concentrations of melamine detected by immunochromatographic strip test combined with anodic stripping voltammetry measurements. The oxidation peak in the voltammograms measured without the strip test was at +1.1 V (Figure 5a), whereas after separation with the strip test the peak shifted to +0.9 V. This was likely due to the influence of the antibody and BSA, which surrounded the AuNP. However, a linear calibration curve from the concentration range of 0–6 mg/L \((R^2 = 0.98)\) could be achieved (Figure 6b) with a sensitivity of 0.19 \(\mu\)A/ppm and an estimated limit of detection (LOD) of 0.1 mg/L \((S/N = 3)\). Better LOD was also shown in comparison to some other methods, as presented in Table 1 [7,19,20]. Furthermore, relative standard deviation (RSD) of ~5% was achieved for standard solution of 0.6 mg/L melamine, indicating good stability of the current responses.

**Selectivity examination of samples containing cyanuric acid and urea, which have similar chemical structures to melamine, showed negative results. Although polyclonal antibody of melamine was utilized in the strip tests [12], several separation stages were involved, including immune selectivity of the antibody, paper chromatography of the strip test, and potential oxidation selectivity of ASV. Moreover, to the best of our knowledge, this is the first electrochemical method applied to the quantification of melamine that utilized immunochromatographic strip tests with AuNP as the label. Therefore, although a strip test using a UV-Vis optical reader shows approximately two orders better LOD, the proposed method suggests a promising alternative for selective detection of melamine.**

**Figure 6. Anodic Stripping Voltammograms Obtained from Immunochromatographic Strip Tests Coupled with ASV Technique at BDD Electrodes (a) and its Concentration Dependence Curve (b). Other Conditions were Similar to those of Fig. 5**

**Table 1. Comparative Performance of AuNP Detection Methods Applied in Immunochromatographic Strip Test for Melamine Detection**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method</th>
<th>LOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>Test-strip reader [19]</td>
<td>260 mg/L</td>
</tr>
<tr>
<td>Milk</td>
<td>Spectrophotometer [7]</td>
<td>2 mg/L</td>
</tr>
<tr>
<td>Milk</td>
<td>Optical reader [20]</td>
<td>(4.47 \times 10^{-3}) mg/L</td>
</tr>
<tr>
<td>Water</td>
<td>Present method</td>
<td>0.1 mg/L</td>
</tr>
</tbody>
</table>
4. Conclusions

Immunochromatographic strip test for melamine was successfully combined with ASV technique using BDD electrodes. Based on the assumption that the concentration of gold nanoparticles used as the label for melamine antibody was representative of the melamine concentration, a linear calibration curve of melamine could be achieved from the concentration range of 0–0.6 mg/L, with an estimated limit of detection of 0.1 mg/L and RSD of ~5%. Selective detection was evidenced by the negative results of strip tests for cyanuric acid and urea, which have similar chemical structures to melamine.

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References