Sensors and Materials, Vol. 33, No. 3 (2021) 1027–1036
MYU Tokyo

S & M 2513

Nickel Hydroxide Nanoparticles for Application in Immunochromatographic Strip Tests of Melamine

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(Received December 14, 2020; accepted February 5, 2021)

Keywords: $Ni(OH)_2$ nanoparticles, immunochromatographic strip test, labels, melamine, biosensors

Nickel hydroxide nanoparticles [$Ni(OH)_2$-NPs] synthesized by a complexation-precipitation method under hydrothermal conditions were employed as a probe in immunochromatographic strip tests for the selective detection of melamine. The characterization of $Ni(OH)_2$-NPs and their conjugation to anti-melamine antibody (anti-MEL) were performed using a UV–visible spectrophotometer, Fourier transform infrared (FTIR) spectroscopy, and TEM. On the basis of the green line intensity, the strip test can selectively detect melamine at a minimum concentration of 25 ppm. Our results suggest that this system is promising for the quantitative detection of melamine.

1. Introduction

Nickel hydroxide [$Ni(OH)_2$] is extensively studied owing to its high theoretical capacity for high-performance battery and supercapacitor systems.[1,2] Recently, the synthesis of $Ni(OH)_2$ nanoparticles [$Ni(OH)_2$-NPs] with controllable sizes ranging from 3.3 to 12.2 nm through the scalable formation and disassociation of a nickel-citrate complex has been reported.[3] These nanoparticles are green, making them eye-catching for application as a probe in optical sensors. Applications of metal nanoparticles as probes or labels in analytical methods have attracted great attention owing to their similar dimensions to biomolecules, such as enzymes, particularly for immunochromatographic strip tests, which combine an immunoreaction with a chromatographic technique, for protein analysis and clinical diagnosis.[4–9]

Meanwhile, gold nanoparticles are the most popular metal nanoparticles applied in immunochromatographic strip tests owing to their distinctive red color and good affinity to proteins.[5] However, although gold nanoparticles possess excellent biocompatibility and stability, they are relatively expensive compared with other common metals such as nickel.[10] In this study, $Ni(OH)_2$-NPs have been synthesized for use as a probe in immunochromatographic strip tests. It is expected that the use of nickel-based nanoparticles will lead to the realization of

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https://doi.org/10.18494/SAM.2021.3214

ISSN 0914-4935
https://myukk.org/

an inexpensive method more suitable for public requirements. As an example of an application, the selective detection of melamine with immunochromatographic strip tests is demonstrated.

Melamine (1,3,5-triazine-2,4,6-triamine) is a nitrogen-rich compound. It has recently been reported that some unethical manufacturers had deliberately added melamine to food to increase the nitrogen content.[11,12] The general method for determining proteins is the Kjeldahl method, and added melamine can be falsely detected as amino acids when determining the protein contents of products. Cases of melamine adulteration in pet food and infant formulas were reported, where the latter resulted in the hospitalization of nearly 54000 infants, at least six of which died.[13,14] Accordingly, many countries such as the USA, Europe, and Australia have set a maximum level for melamine in food of 2.5 mg/kg, while China has set a maximum level of 1 mg/kg for infant formulas and 2.5 mg/kg for milk as well as food containing milk products.[13,14]

In this study, we developed immunochromatographic strip tests for the detection of melamine[5] as alternatives to, for example, chromatography,[11,12,15] capillary electrophoresis,[16] enzyme-link immunosorbent analysis (ELISA),[17] chemiluminescence,[18] and fluorescence.[19] Our investigation of $Ni(OH)_2$-NPs as a probe in immunochromatographic strip tests of melamine showed that the selective quantitative detection of melamine can be performed, suggesting that $Ni(OH)_2$ nanoparticles are promising for such applications.

2. Materials and Methods

2.1 Materials

Melamine ($C_3H_6N_6$), nickel nitrate [$Ni(NO_3)_2$], sodium borohydride ($NaBH_4$), and trisodium citrate ($Na_3C_6H_5O_7$) were from Wako Pure Chemical Industries (Japan), while nitrocellulose membrane (5 μm pore size), 2-chloro-4,6-diamino-1,3,5-triazine (CAAT), γ-aminobutyric acid (GABA), N-hydroxysuccinimide (NHS), dimethylformamide (DMF), N,N′-dicyclohexylcarbodiimide (DCC), bovine serum albumin (BSA), Freund's Complete and Incomplete Adjuvants, and other chemicals were from Sigma-Aldrich. Liquid Blocker Super PAP Pen Mini and plastic backing were supplied by Daido Sangyo, Japan. Double-distilled water with a maximum conductivity of 18 MΩ was obtained from a Simply-Lab water system (DIRECT-Q 3 UV, Millipore).

2.2 Synthesis and characterization of nickel hydroxide nanoparticles [$Ni(OH)_2$-NPs]

$Ni(OH)_2$-NPs were synthesized in accordance with Ref. 3 with slight modification. A solution of $Ni(NO_3)_2$ was mixed with 10 mL of 0.1 M $NaBH_4$ solution. Then, $C_6H_5Na_3O_7$ solution was added in various ratios to the $Ni(NO_3)_2$ as shown in Table 1. After stirring at 1500 rpm at 35 °C for 10 h, 5 mL of 4 M NaOH was added to the mixture, which was then stirred for 5 min before heating in an autoclave at 120 °C for 24 h. Characterization was performed using UV–visible (UV–Vis) and Fourier transform infrared (FTIR) spectrophotometers as well as by TEM.

Table 1
Mixtures of $Ni(NO_3)_2$ and $C_6H_5Na_3O_7$ used for the synthesis of $Ni(OH)_2$-NPs.

Variant	Total concentration (M)	
	$Ni(NO_3)_2$	$C_6H_5Na_3O_7$
A	4.91×10^{-5}	3.31×10^{-4}
B	4.91×10^{-4}	3.31×10^{-3}
C	2.46×10^{-3}	1.66×10^{-2}
D	4.91×10^{-3}	3.31×10^{-2}
E	1.23×10^{-2}	8.29×10^{-2}
F	2.4×10^{-2}	1.66×10^{-1}
G	3.6×10^{-2}	2.49×10^{-1}
H	4.91×10^{-1}	3.31×10^{-1}

2.3 Preparation of polyclonal anti-melamine antibody (anti-MEL)

Anti-MEL was produced in our laboratory by injecting the antigen, composed of 4-(4,6-diamino-1,3,5-triazine-2-ylamino) butanoic acid hapten conjugated to BSA, into rabbits.[20] Briefly, the hapten was prepared from 60.5 mg of CAAT dissolved in 75 mL of absolute ethanol. Then, 5 mL of ethanol containing 0.48 g of GABA and 0.82 g of KOH was added dropwise. The mixture was next refluxed at 70 °C for 24 h. The white precipitate obtained after cooling the filtrate was then dissolved and recrystallized.

The hapten was conjugated to BSA by dissolving 20.0 mg of hapten and 17.0 mg of NHS in 1 mL of dimethylformamide, followed by the addition of 31.0 mg of DCC. After overnight stirring at 4 °C, the white precipitate of dicyclohexylurea was removed by centrifugation, while 900 µL of the supernatant was added dropwise to 113 mg of BSA dissolved in 8 mL of 10 mM PBS at pH 7.4. The conjugate mixture was stirred at 4 °C for 12 h and dialyzed against 10 mM PBS at pH 7.4.

The hapten-conjugated BSA was then immunized to a New Zealand rabbit (1.5 kg weight) by injecting 250 µg of hapten-conjugated BSA in 150 µL of Freund's Complete Adjuvant five times at intervals of 14 days. A rabbit blood sample was investigated using an indirect ELISA to confirm the presence of antibodies in melamine. After 40 days, a positive result was obtained and the serum was harvested after 48 days. Prior to use, the serum was melted and filtered using Protein A. Then, it was labeled as anti-MEL.

2.4 Preparation of $Ni(OH)_2$-NPs conjugated to anti-MEL

A 1 mL volume of $Ni(OH)_2$-NPs was centrifuged at 10000 rpm for 20 min and the supernatant was resuspended in 1 mL of 0.01 M PBS at pH 7. After stirring to homogenize the mixture, the mixture was centrifuged and 1 mL of 20 mg/mL anti-MEL was added, which was followed by incubation at 25 °C and 650 rpm for 30 min. Subsequently, 5% BSA was added and the mixture was incubated before the addition of 200 mL of PBS and 200 mL of 10% sucrose. The mixture was homogenized by stirring and then stored in a refrigerator for future usage. The characterization was performed using a UV–Vis spectrophotometer, TEM, and the zeta potential.

2.5 Preparation of immunochromatographic strip tests for melamine detection

The immunochromatographic strip tests were composed of a sample pad, conjugate pad, nitrocellulose membrane, test zone, and absorbent pad, as shown in Fig. 1. The sample pad was prepared from a piece of nitrocellulose membrane dipped into 0.01 M PBS at pH 7.4 containing 5% BSA and 0.05% Tween 20, and dried at 60 °C for 2 h. The conjugate pad was made of fiberglass spiked with $Ni(OH)_2$-NP-conjugated anti-MEL, while the test zone was prepared from a nitrocellulose membrane spiked with 1 mg/mL anti-MEL. Both the conjugated and test zones were dried at 37 °C for 2 h. The strip tests were performed by the dropwise addition of 100 μL of standard melamine solution to the sample pad, followed by incubation for 15 min.

2.6 Electrochemical measurements

Electrochemical measurements were performed by anodic stripping voltammetry (ASV). A solution of 0.1 M $HClO_4$ was used as the electrolyte, while boron-doped diamond (BDD), a Pt wire, and a Ag/AgCl system were employed as working, counter, and reference electrodes, respectively. A deposition potential of −0.6 V, a deposition time of 90 s, and a scan rate of 100 mV/s were applied in the ASV measurements, following previous results.[21,22]

3. Results and Discussion

3.1 Preparation of $Ni(OH)_2$-NPs and $Ni(OH)_2$-NPs conjugated to anti-MEL

$Ni(OH)_2$-NPs were synthesized by the complexation-precipitation method under hydrothermal conditions.[2] Basically, Ni^{2+} ions were reduced to Ni^0 nanoparticles by $NaBH_4$ in the presence of citrate as the capping agent. The reduction of nickel was indicated by the change in the solution color from green to black. The nickel nanoparticles, which were stabilized by citrate, were then re-oxidized to form a Ni^{2+}-citrate complex. A hydrothermal process was performed to eliminate the interaction of Ni-citrate to form crystal seeds, which gradually grew through the dissolution-crystallization mechanism to become $Na(OH)_2$ nanocrystals in the presence of NaOH as the hydroxyl source.[3] Various concentrations of citrate were investigated to optimize the nickel:citrate ratio. The UV–Vis spectra [Fig. 2(a)] show that the $Ni(OH)_2$-NP

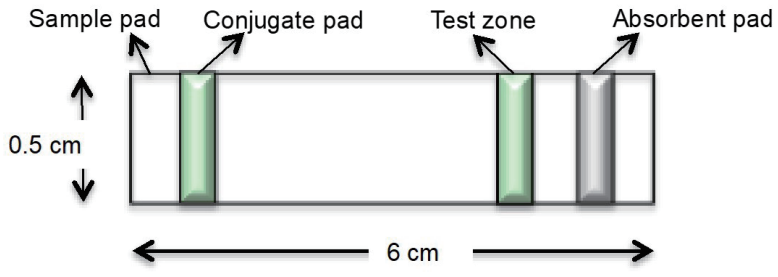
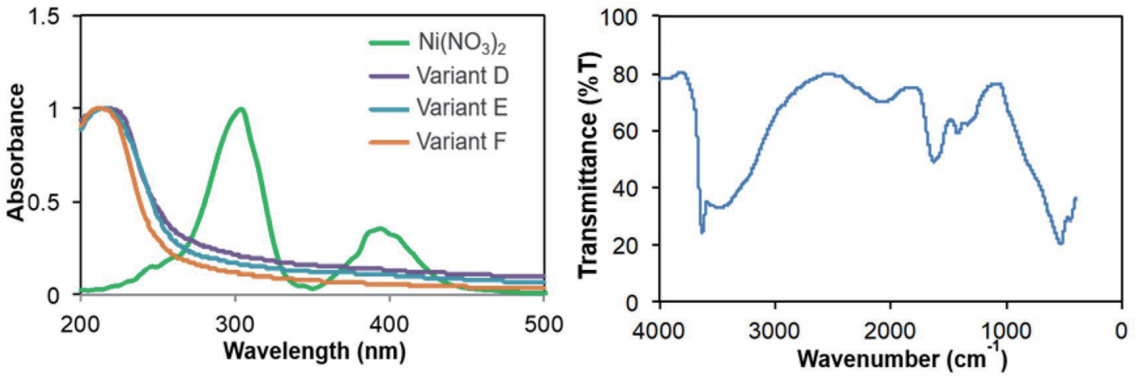


Fig. 1. (Color online) Schematic of the immunochromatographic strip test.



(a) (b)

Fig. 2. (Color online) (a) UV–Vis spectra of $Ni(NO_3)^2$ solution in comparison with that of $Ni(OH)_2$-NPs and (b) FTIR spectrum of $Ni(OH)_2$-NPs.

spectrum is different from that of $Ni(NO_3)_2$, which acts as the precursor of the $Ni(OH)_2$-NPs. The spectrum, which was investigated to follow the reaction, indicates that only compositions (D), (E), and (F) in Table 1 satisfied the criterion of $Ni(OH)_2$-NPs of an absorption peak at 220–230 nm.[2] The results suggested that a nickel/citrate ratio of around 7 to 70 is best for synthesizing $Ni(OH)_2$-NPs. The composition with the highest peak intensity [composition (F)] was then selected for the next step because a more homogeneous color, shown by its sharp peak, is necessary for the probe in immunochromatographic strip tests.

The FTIR spectrum of $Ni(OH)_2$-NPs shown in Fig. 2(b) reflects broad absorption at a wavenumber of around 3600 cm^{-1}, indicating the stretching vibration of O–H. A sharp peak at 3624 cm^{-1}, related to the absorption band of the O–H stretching vibration that binds to nickel, was also observed. In addition, bands at 1650 and 1400 cm^{-1}, attributed to the absorption bands of C=O asymmetrical stretching and symmetrical stretching, respectively, were also observed. This C=O group is assumed to be from the carbonyl group in the citrate ions used as a capping agent, which were still present in the nanoparticles. The symmetrical carbonyl peak was probably due to the presence of citrate ions in the sample. Moreover, no peaks at 3444.98, 435.93, and 474.5 cm^{-1} were observed, which would have been attributable to the formation of NiO. Furthermore, the $Ni(OH)_2$-NPs were also characterized by XRD, which revealed that the $Ni(OH)_2$-NPs had a chemical formula of $H_2Ni_1O_2$ and exhibited a hexagonal phase (space group number: 166; a: 3.0710; b: 3.0710; c: 23.2000). Thus, according to the XRD patterns, the $Ni(OH)_2$-NPs were successfully formed.

The TEM image in Fig. 3(a) shows that the $Ni(OH)_2$-NPs have a hexagonal structure with a size between 14 and 17 nm. The figure shows that some particles are flat and some are side-stacked, confirming the TEM results of ß-$Ni(OH)_2$ nanoparticles prepared by Wang *et al.* and Mao *et al.*[2,3] Wang *et al.* depicted the hexagonal ß-$Ni(OH)_2$ as a lamellar structure with hydrogen atoms located between NiO_6 octahedra.[3]

The $Ni(OH)_2$-NPs were conjugated to anti-MEL by an electrostatic interaction because neutral colloidal $Ni(OH)_2$-NPs capped by negatively charged citrate will bind to the positive charge of the amino acid.[5] The antibody is a zwitterion, which means that it has both positive

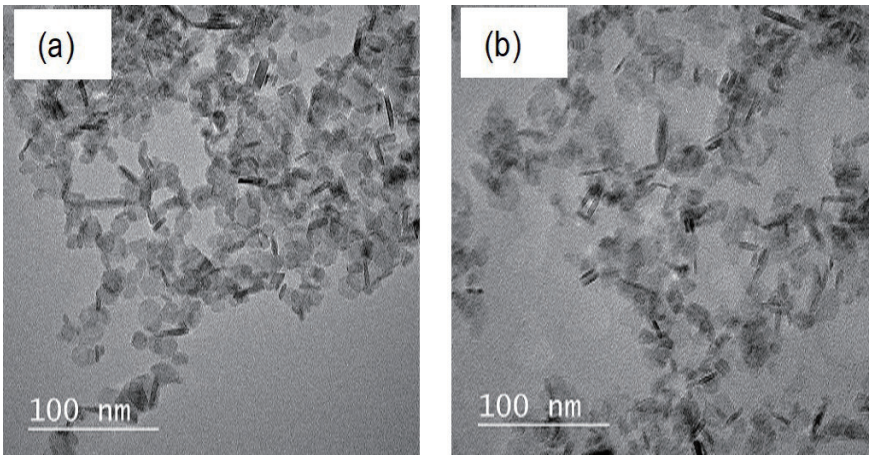


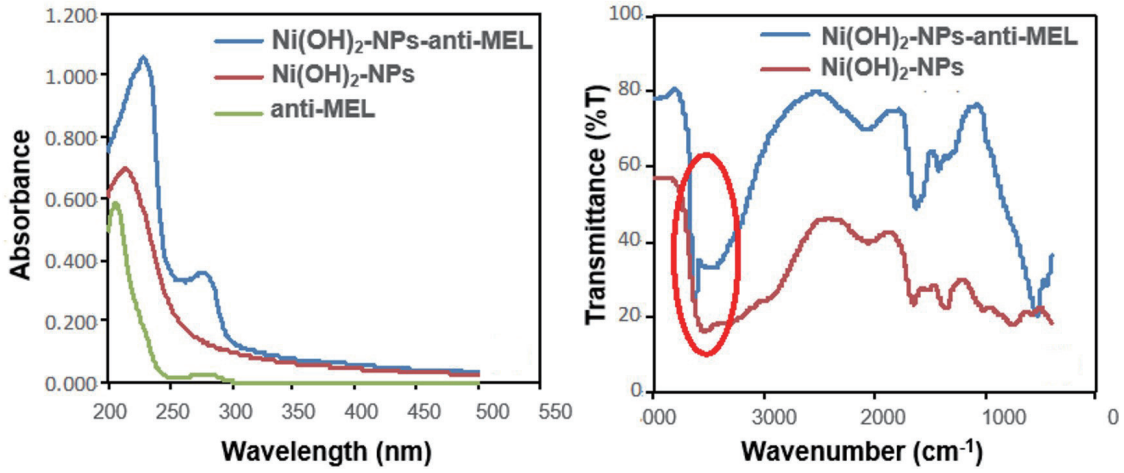
Fig. 3. TEM images of $Ni(OH)_2$-NPs (a) without and (b) with conjugation to anti-MEL.

and negative charges. The negative charge of the amino acid residue will bind specifically to melamine. In addition, melamine is known to have a pKa of 5, allowing it to bind with amino acid residues of negatively charged alkaline antibodies. Bio-conjugation between $Ni(OH)_2$-NPs and anti-MEL is expected to occur non-covalently due to the adsorption of antibody molecules on the surface of the nanoparticles through the electrostatic interaction of the antibody with the nanoparticles.

The anti-MEL was synthesized by injecting melamine into rabbits. Since melamine is not a protein, no antibody will be produced if only melamine is injected into the living body. Therefore, melamine has to be modified with protein before injection. In this case, BSA was used. To increase the affinity of melamine to protein, hapten was prepared as a bridge between melamine and protein. The interactions between protein, hapten, and melamine may occur because the surface of nanoparticles has negative charges due to the presence of citrate as the capping agent, while the antibody exhibits more positive charges.[5] BSA is also necessary for the immunochromatographic system by acting as a blocking agent on the surface of the nanoparticles to prevent the adsorption of specific proteins.[23,24]

The TEM image [Fig. 3(b)] shows that the nanoparticles are less aggregated than those in Fig. 3(a), suggesting that the nanoparticles are affected by the presence of anti-MEL. It is considered that the negatively charged nanoparticles are attracted to the positive charges of the amino acid residues of the anti-MEL at different locations; hence, they become more evenly distributed.

The UV–Vis spectra [Fig. 4(a)] showed the absorbance peak of $Ni(OH)_2$-NPs at around 220 nm, whereas one of the antibodies exhibited an absorption peak at 280 nm. After conjugation with anti-MEL, a slight redshift of the former peak to 230 nm was observed, indicating an interaction between the $Ni(OH)_2$-NPs and the anti-MEL. The IR spectrum in Fig. 4(b) shows the loss of the peak intensity at 400 cm^{-1} and a strong, broad peak at 3000–3600 cm^{-1}, which indicates that the O–H hydroxyl group turns into an O–H carboxylic group. The carboxylic group is assumed to be derived from antibodies conjugated to



(a) (b)

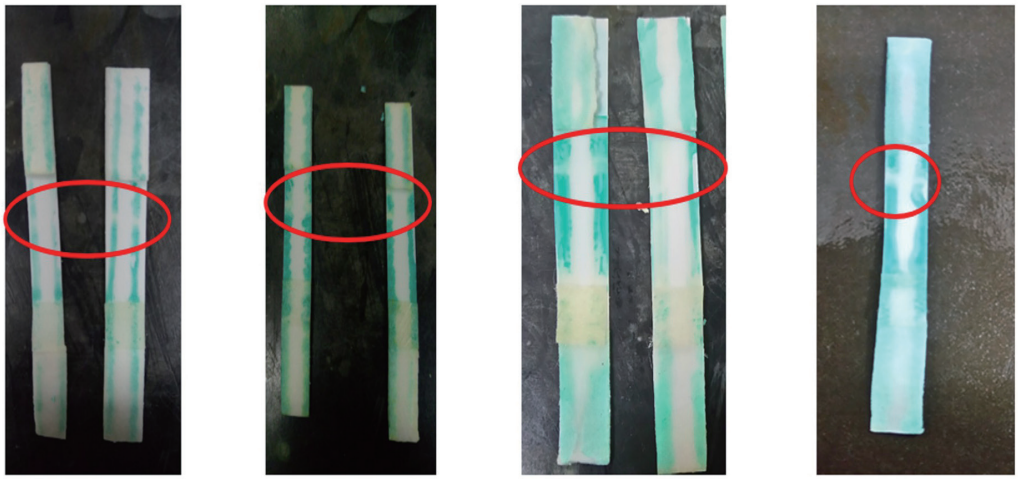
Fig. 4. (Color online) (a) UV–Vis spectra of $Ni(OH)_2$-NPs, $Ni(OH)_2$-NPs-anti-MEL, and purified anti-MEL. (b) IR spectra of $Ni(OH)_2$-NPs and $Ni(OH)_2$-NPs-anti-MEL.

$Ni(OH)_2$-NPs. In addition, the potential surface of the $Ni(OH)_2$-NPs examined by the zeta potential was found to be more positive (−2.53 mV) after conjugation with anti-MEL than the unconjugated nanoparticles (−19.94 mV), suggesting that the surface of the nanoparticles changed from citrate to anti-MEL, which is more positive.

3.2 Application of $Ni(OH)_2$-NPs on immunochromatographic strip test for melamine detection

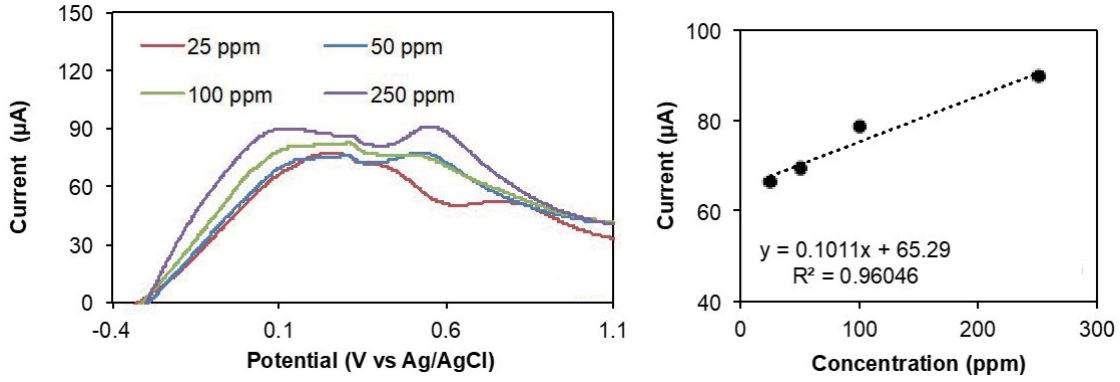
An immunochromatographic strip test was performed to evaluate the application of $Ni(OH)_2$-NPs as a probe. Melamine standard solutions of 25, 50, 100, and 250 ppm were used as the samples. Samples interacted and were specifically captured by anti-MEL that had been conjugated to $Ni(OH)_2$-NPs in the conjugated pad. Then, the formed complexes moved along the nitrocellulose membrane by capillarity to reach the test zone. In the test zone, the complex was captured by the anti-MEL that had been previously placed to form a sandwich complex of anti-MEL–$Ni(OH)_2$-NPs–anti-MEL.[4,5] Positive results were indicated by a distinct green line in the test zone (Fig. 5), which occurred at a minimum concentration of 25 ppm. The intensity of the green line in the test zone increased with the concentration of melamine, indicating the successful development of a strip test in which $Ni(OH)_2$-NPs are applied as the probe.

The green line was then cut and dissolved in 0.1 M $HClO_4$, which was used as a supporting electrolyte solution, and the electrochemical measurements were performed by ASV at the BDD electrode. Voltammograms of the measurements at a deposition potential of −600 mV, a deposition time of 90 s, and a scan rate of 100 mV/s are shown in Fig. 6. An oxidation peak was observed at a potential of +0.1 V [Fig. 6(a)]. The potential of the oxidation peak shifted from the original value of 0.0 V for $Ni(OH)_2$-NPs to +0.1 V for $Ni(OH)_2$-NPs–anti-MEL, while the oxidation peak at the potential of +0.6 V is assumed to be due to one of the amino acids contained in the antibody (cysteine according to a previous study)[25,26] rather than melamine.



(a) (b) (c) (d)

Fig. 5. (Color online) Photographs of immunochromatographic strip test for detection of melamine at concentrations of (a) 25, (b) 50, (c) 100, and (d) 250 ppm.



(a) (b)

Fig. 6. (Color online) (a) Anodic stripping voltammograms of anti-MEL-Ni(OH)$_2$-NPs–anti-MEL at various concentrations and (b) linear calibration curve.

From the linear calibration curve [Fig. 6(b)], there is a linear relationship between the current and concentration in the concentration range of 25–250 ppm (R^2 = 0.9605) with a detectable melamine concentration limit of the test strip of 53.90 ppm.

4. Conclusions

Ni(OH)$_2$-NPs have been successfully synthesized with an average size of around 14–17 nm. The application of the nanoparticles conjugated to anti-MEL as a probe in an immunochromatographic strip test for melamine detection showed positive results in the form of a green line in the test zone. Furthermore, a preliminary study on the quantitative detection of melamine using ASV at a BDD electrode suggested that the conjugated Ni(OH)$_2$-NPs with

anti-MEL are promising for quantitatively detecting melamine and can be potentially applied in food processing and health industries.

Acknowledgments

This research was funded by Hibah Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) 2020 from the Ministry of Education and Culture, Republic of Indonesia (contract No. NKB-2814/UN2.RST/HKP.05.00/2020).

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