Development of an antibody-based colloidal gold immunochromatographic lateral flow strip test for natamycin in milk and yoghurt samples

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ABSTRACT

An immunochromatographic lateral flow strip test was developed for the detection of natamycin (Nata) residues in milk and cheese samples. Monoclonal antibody (mAb) against Nata was produced with a half maximal inhibitory concentration of 1.85 \( \mu \text{g L}^{-1} \). MAb conjugated with gold nanoparticles was the detection reagent. Nata conjugated with ovalbumin via the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide method immobilized on a nitrocellulose membrane was the capture reagent. This semi-quantitative method required only 15 min to complete. The optimum concentrations of coating antigen and mAb were 0.15 \( \mu \text{g mL}^{-1} \) and 0.2 \( \mu \text{g mL}^{-1} \), respectively. Based on an optical density scanner, the visual limit of detection of Nata was 5 \( \mu \text{g L}^{-1} \) and 10 \( \mu \text{g kg}^{-1} \) in milk and yoghurt samples, respectively.

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Introduction

Natamycin (Nata) is a macrolide polyene antifungal agent produced during the fermentation activity of Streptomyces natalensis, S. chmanovgensis, S. gilvosporeus, etc. (Chen, Lu, & Du, 2008; Sun et al., 2016). Nata was mainly used in medicine after its discovery, it has gradually become incorporated into food preservatives (El-Enshasy, Farid, & El-Sayed, 2000) (Fajardo et al., 2010; Hondrodimou, Kourkoutas, & Panagou, 2011). In the food industry, Nata is commonly used as a preservative in milk and yoghurt (Dervisoglu, Gul, Aydemir, Yazici, & Kahyaoglu, 2015), cheese (Fuselli et al., 2012), ham (Camilloto, Soares, Pires, & de Paula, 2009), sausage (Pintado, Ferreira, & Sousa, 2010). Due to their potential risk on consumer health, the use of food additives including antimicrobial preservatives is strictly regulated by national and international authorities. In early years, Nata was allowed as a food additive only by the Food and Agriculture Organization of the United Nations/World Health Organization and the United States Food and Drug Administration and China (El-Enshasy et al., 2000). Currently, Nata is used in more than 30 countries. Even though Nata has several advantages including eurytopicity, high efficiency, and low toxicity, a study has reported that Nata induces nausea, diarrhea, and anorexia among other symptoms (Repizo, Martinez, Olsina, Cerutti, & Raba, 2012). Additionally, the daily ingestion of Nata may weaken the
immune system (Repizo et al., 2012). Therefore, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives regulated that the acceptable daily intake of Nata is 0.3 mg kg\(^{-1}\) body weight (EFSA, 2009; EU, 1995).

There are some reports about the analysis of Nata residues. Most of the methods were depend on the instruments such as high-performance liquid chromatography–mass spectrometry (HPLC–MS) (Vierikova, Hrnciarikova, & Lehotay, 2013; Zhou, Zhu, Xia, He, & Zhang, 2014), LC–MS/MS (Molognoni, Ploencio, Valese, Lindner, & Daguer, 2016; Molognoni, Valese, Lorenzetti, Daguer, & Lindner, 2016), and HPLC-diode array detector (Paseiro-Cerrato et al., 2013). Even though they are highly sensitive and specific, these methods require expensive instruments, time-consuming sample pretreatments, and trained technicians. Additionally, these methods are not suitable for the high-throughput and rapid screening of samples.

Immunoassays such as the enzyme-linked immunosorbent assay (ELISA) have been extensively applied in the food safety detection. In particular, indirect and competitive ELISA (ic-ELISA) was suitable for the detection of small molecules, including antibiotics (Adrian, Fernandez, Sanchez-Baeza, & Marco, 2012), hormones (Kong et al., 2015), heavy metals (Wang et al., 2012), and pesticides residues (Chen et al., 2013). The ic-ELISA (working range: 0.64–4.46 μg L\(^{-1}\)) based on monoclonal antibody (mAb) has been also established for detection of Nata residues in foods (Chen et al., 2015). Compared with instrumental methods, ic-ELISA is more rapid, simpler, and adequate for the high-throughput screening. However, the complex operations (washing, incubation, etc) were involved during ic-ELISA. In contrast, colloidal gold immunochromatographic lateral flow strip was more convenient without those complex steps. The whole test can be fulfilled in 10–15 min. The lateral flow strip test has also been applied in food safety detection programs (Guo et al., 2015; Preechakasedkit et al., 2012). To the best of my knowledge, no study has used the colloidal gold lateral flow strip test for the detection of Nata residues. In this study, a sensitive mAb-based lateral flow strip was established for the Nata detection in milk and yoghurt samples.

**Materials and methods**

**Reagents**

Natamycin hydrochloride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and \(N\)-Hydroxysuccinimide (NHS) were purchased from J&K Scientific Ltd. (Beijing, China). Bovine serum albumin (BSA), ovalbumin (OVA), Freund’s complete and incomplete adjuvant, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), 25% glutaraldehyde (GA) solution, and 3,3’,5,5’-tetramethylbenzidine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Goat anti-mouse immunoglobulin (IgG) antibody was acquired from Jackson ImmunoResearch Laboratories (PA, USA). All other chemicals and solvents were analytical grade.

Pure water in the whole experiment was prepared using the milli-Q ultrapure system (Bedford, MA, USA). Polyvinylchloride (PVC) pads, absorbance pad (H5079), and the sample pad (GL-b01) were obtained from JieYi Biotechnology Co., Ltd. (Shanghai, China). The nitrocellulose (NC) membrane (Unistart CN140) was obtained from Sartorius Stedim Biotech GmbH (Goettingen, Germany). The CM4000 Guillotine Cutting Module and the Dispensing Platform were acquired from Kinbio Tech Co., Ltd. (Shanghai, China).
**Preparation of mAb against Nata**

Immunogen (Nata-GA-BSA) and coating antigen (Nata-EDC-OVA) were synthesized as previously reported (Chen et al., 2015). The synthesis routine of immunogen and coating antigen is shown in Figure 1. The immunogen was subcutaneously injected into mice with multiple dots. The immunization schedule was performed according to reference (Xu et al., 2016). The first immunization administered with immunogen emulsified with Freud’s complete adjuvant. Subsequent booster immunizations were administered with immunogen emulsified with Freud’s incomplete adjuvant. After the fifth immunization, the mouse with the highest titer and lowest inhibition against Nata was selected to donate spleen for cell fusion. A booster intra-peritoneal injection (20 µg of immunogen dissolved in 100 µL physiological saline) was administered to the mouse three days prior to cell fusion. Seven days after cell fusion, ic-ELISA was performed to detect the supernatant of hybridomas to screen the positive wells towards Nata. The selected hybridomas were sub-cloned three times to obtain a pure cell line. The pure cell lines were cultivated in a large scale and the supernatant of each cell line was intraperitoneally injected into mice primed with paraffin to obtain ascites, Ascites were purified by the saturated-ammonium-sulfate method to get pure mAbs. The concentration of mAbs was measured at 278 nm by UV–VIS spectrometry.

**Preparation of colloidal gold-mAb conjugates**

Gold nanoparticles were synthesized with some modifications according to previously reported (Kong et al., 2016). Briefly, 50 mL of HAuCl₄·4H₂O (0.01%, w/v) was boiled thoroughly under constant stirring and rapidly added with 2 mL of freshly prepared 1% (w/v) trisodium citrate. The mixture solution was boiled until the color of the solution changed to wine-red. Then, the solution was cooled to room temperature and stored at 4°C for future use. Gold nanoparticles were characterized by transmission electron microscopy (TEM) and UV–VIS spectrometry.

The colloidal gold were conjugated with mAb via electrostatic interaction (Chen et al., 2017). Briefly, 80 µL of K₂CO₃ (0.1 M) was added into 20 mL of colloidal gold to adjust pH

Figure 1. The synthesis of immunogen and coating antigen.
to 9. MAb (0.2 mg) were slowly added into the colloidal gold solution under continuous stirring for 30 min at room temperature. BSA (50 mg) dissolved in 2 mL of ultrapure water was added into the colloidal gold-mAb solution under constant stirring for 30 min. The solution was centrifuged at 875 rpm for 40 min to remove free blocking agent and excess mAb. The resulting precipitate was re-suspended twice in borate buffer (0.002 M, pH 8, containing 1% (w/v) sucrose and 0.01% Tween-20).

**Fabrication of the lateral flow strip**

As shown in Figure 2(A), from the cross-section view, the lateral flow strip consist of five sections: a plastic backing pad, a NC membrane, a sample pad, a conjugate pad, and an absorbent pad (Xing et al., 2015). As shown in Figure 2(B), the control line and test line on the NC membrane were coated with different capture reagents, respectively. The coating antigen (Nata-EDC-OVA) previously diluted to 0.15 μg/mL was immobilized on test line, while goat anti-mouse IgG was dispensed on the control line. Colloidal gold-mAb was coated on the conjugate pad. The membranes were dried for 1 h at room temperature, assembled as shown in Figure 2(A), sealed in plastic bag, and stored at 4°C.

**The principle of the lateral flow strip**

The principle of the lateral flow strip test is similar to principle of ic-ELISA (Liu, Peng, Jin, & Xu, 2007; Peng et al., 2017). Standard solution or sample extract is added in the microtiter plate. The end of the sample pad is inserted into the well for 10 s and rapidly wetted through. Due to the capillary effect, colloidal gold-mAb immobilized on the conjugate pad dissolved and flows with the sample to the NC membrane. The strip is put flat to allow the solution to transfer more smoothly. Colloidal gold-mAb can conjugate with goat anti-mouse IgG in the control line; therefore, regardless of the presence of analytes, one red

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**Figure 2.** Schematic image of lateral flow strip test: (A) cross-section; (B) top view.
band consistently appears on the control line, certifying the validity of the test. If the sample is Nata-negative, the coating antigen immobilized on the test line is captured by the colloidal gold-mAb, forming a red band. In contrast, if the samples are Nata-positive, the colloidal gold-mAb is conjugated with Nata soon. The coating antigen in test line competes with colloidal gold for the binding with Nata. Therefore, the test line will be colorless in Nata-positive samples. Generally, the weaker test line is, the higher concentration of Nata in samples is. The concentration of analyte that results in an obvious difference between the test line and control line is defined as the visual limit of detection (vLOD).

**Analysis of milk and yoghurt samples using lateral strip test**

Nata-negative milk and yoghurt samples were spiked with different concentrations of Nata previously dissolved in dimethylformamide at 1 mg mL\(^{-1}\). The milk samples were diluted 10 times by HEPES (0.01 M, pH 7.2) to eliminate matrix interference. The concentrations of Nata in the spiked milk samples were 0, 1, 2, 5, 10, and 20 µg L\(^{-1}\). Homogenized yoghurt (2 g) was transferred into polypropylene centrifuge tubes and mixed with 20 mL of methanol. The tubes were shaken on an orbital shaker for 20 min. Deionized water (25 mL) was added, and the tubes were stored at 4°C for 30 min. The extract was passed through a filter paper, and the resulting filtrate was passed through a 0.2-µm membrane microfilter. For yoghurt sample, the final concentration of Nata was 0, 2, 5, 10, 20, and 25 µg L\(^{-1}\).

A standard curve for Nata was developed for strip test based on the \(T/T_0\) value, which were determined by a hand-held scanner. The \(T_0\) value and \(T\) value represent the chromaticity of the control line and test line. The \(T/T_0\) ratio is indicative of the difference between the control line and test line, which is important for evaluating the vLOD of the strip test.

**Results and discussions**

Nata has been commonly used in the food industry. The shelf-life of yoghurt is relatively short. Therefore, preservatives are commonly added to yoghurt. The excessive use of additives in foods is banned.
Pretreatments of samples

In general, liquid samples are easily prepared for immunoassays. To eliminate matrix effects, milk samples were only diluted. Yoghurt, a semi-solid food that contains carbohydrates, proteins, amino acids, and lipids, is more complex. The solubility of Nata is higher in methanol than in water. Methanol denatures protein, leading to protein precipitation. In methanol, amino acid solubility is poor and carbohydrates cannot be dissolved. Lipids are separated out under frozen conditions; therefore, the yoghurt samples were placed at 4°C for 30 min.

Optimization of the lateral flow strip test

In the presence of trisodium citrate, HAuCl₄ was reduced into gold nanoparticles. Due to electrostatic interaction, gold nanoparticles form a stable hydrophobic solution that is negatively charged, which is called colloidal gold. The negatively charged colloidal gold solution and positively charged proteins bind tightly under slightly alkaline conditions. Therefore, the K₂CO₃ was used to adjust the pH of colloidal gold. The TEM image and UV–VIS spectrum of colloidal gold were shown in Figure 3.

Figure 4. The images of strip tests for Nata spiked in milk samples and yoghurt samples. Milk samples: (1)0 µg L⁻¹, (2)1 µg L⁻¹, (3)2 µg L⁻¹, (4)5 µg L⁻¹, (5)10 µg L⁻¹, and (6)20 µg L⁻¹. Yoghurt samples: (1)0 µg kg⁻¹, (2)2 µg kg⁻¹, (3)5 µg kg⁻¹, (4)10 µg kg⁻¹, (5)20 µg kg⁻¹, and (6)25 µg kg⁻¹.
The concentration of coating antigen and mAb is important for the test. Different concentrations of coating antigen (0.05, 0.15, 0.3, and 1 µg mL\(^{-1}\)) and mAb (0.1, 0.2, 0.4, and 0.8 µg mL\(^{-1}\)) were evaluated to screen the optimum conditions. The results revealed that the optimum concentrations of coating antigen and mAb were 0.15 µg mL\(^{-1}\) and 0.2 µg mL\(^{-1}\), respectively.

**Determination of lateral flow strip test results**

The lateral flow strip test results were visually determined based on the color intensity of the test and control lines. The color intensity of the test line is inversely proportional to the concentration of Nata. The higher the Nata concentration in the sample, the weaker the color of the test line. When the Nata concentration in the sample is below the detection limit, the difference between the color intensity between the two lines cannot be distinguished by the naked eye. When no color appears on either line, the test is considered to be invalid.

**Detection limit of strip test for Nata**

The main advantage of the lateral strip assay was that the results can be visually analyzed. The color intensity of the test line must be strong enough to be seen and to identify a difference between negative control and samples. As shown in Figure 4, milk samples spiked with 5 µg L\(^{-1}\) Nata resulted in a slight but distinguishable difference between the control and test lines. Therefore, the vLOD of Nata in milk samples was 5 µg L\(^{-1}\). Similarly, the vLOD of Nata spiked in yoghurt samples was 10 µg kg\(^{-1}\). Additionally, vLOD can be calculated from the standard curve (Figure 5) of Nata-spiked milk and yoghurt samples.

![Figure 5](image.png)

**Figure 5.** The standard curve for Nata spiked in milk and yoghurt samples.
**Conclusions**

The major advantages of the lateral flow strip assays are that results can be obtained within 15 min by the naked eyes and that it requires simple sample pretreatment. The strips could be used for on-site screening of Nata residues in milk and yoghurt samples. The assay provides a preliminary and semi-quantitative method for high-throughput and one-site determination.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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