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NOTE

A highly sensitive europium nanoparticle-based lateral flow immunoassay for detection of chloramphenicol residue

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Abstract A europium nanoparticle-based lateral flow immunoassay for highly sensitive detection of chloramphenicol residue was developed. The detection result could be either qualitatively resolved with naked eye or quantitatively analyzed with the assistance of a digital camera. In the qualitative mode, the limit of detection (LOD) was found to be 0.25 ng/mL. In the quantitative mode, the half-maximal inhibition concentration (IC50) was determined to be 0.45 ng/mL and the LOD can reach an ultralow level of 0.03 ng/mL, which is ~100 times lower than that of the conventional colloidal gold-based lateral flow immunoassay. Potential application of the established method was demonstrated by analyzing representative cow milk samples.

Keywords Detection · Chloramphenicol · Lateral flow immunoassay · Europium chelate · Nanoparticle

Introduction

Chloramphenicol (CAP) is an effective broad-spectrum antibiotic and is widely used in veterinary practices. Nevertheless, CAP is prohibited in many countries since it causes serious side-effects in humans, such as bone marrow depression and aplastic anemia [1–3]. A permanent control of CAP levels in foodstuffs of animal origin is, therefore, indispensable. To this end, numerous analytical techniques have emerged to monitor the presence of CAP in food samples, including enzymatic and chromatographic [4–6]. However, these methods always involve multiple processing steps and rely on large-scale equipments, making them less possible to be used in on-site (or outside the laboratory) detection of CAP. In contrast, membrane-based lateral flow immunoassay (LFIA) technique is specifically designed for the on-site detection because of its simplicity and rapidness [7, 8]. LFIA has been utilized for detection of a myriad of analytes from small molecules to macromolecules and microorganisms. Despite these demonstrations, implementing LFIA into detection of CAP remains to be a grand challenge because of the requirement of extremely low limit of detection (LOD) by various countries/unions (e.g., a LOD of 0.3 μg/kg or ng/mL was required by the European Union in 2002) [5, 9]).

We have recently reported an ultrasensitive LFIA based on the use of europium (Eu) (III) chelates-loaded silica nanoparticle as the label, with which a low LOD of 0.03 ng/mL was achieved for hepatitis B surface antigen (HBsAg) in a 'sandwich' detection mode [10]. The high analytical sensitivity of this LFIA mainly benefited from: (1) high fluorescence intensity of the Eu (III) nanoparticle label because of the multilayer loading of Eu chelates onto the porous silica nanoparticles; and (2) low background fluorescence owing to the long-wavelength

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emission signal of Eu chelates (λ_{em}=615 nm) [10, 11]. In the present study, we further extend the capability of this ultrasensitive LFIA to detect small-molecule CAP using a 'competitive' detection mode.

Methods

Figure 1 schematically shows the principle of the competitive LFIA of CAP. The nanoscale labels (i.e., conjugations of Eu chelates-loaded silica nanoparticle and anti-CAP polyclonal antibodies via the linkage of oxidized dextran) were dispersed in the conjugate pad. CAP-bovine serum albumin (CAP-BSA) conjugates and goat-anti rabbit secondary antibodies were immobilized on the test- and control-line areas of the membrane, respectively. The anti-CAP polyclonal antibodies were raised in New Zealand white rabbits using CAP-keyhole limpet hemocyanin (CAP-KLH) conjugates as the immunogen. The selectivity of the anti-CAP polyclonal antibodies was proven to be considerably high as shown by the cross-reactivity experiments. The cross-reactivities for other fenicol drugs (analogues of CAP), thiamphenicol and florfenicol, were determined to be less than 0.01 %, and BSA was ~0.03 % (see Electronic Supplementary Material and Table S2c for details). During the detection process, liquid sample was dropped onto the sample pad of the test strip. If a large amount of CAP is present in the sample, CAP will neutralize the nanoparticle-labeled antibodies, leaving fewer binding sites available for the CAP-BSA conjugate at the test line. As a result, there will be a small amount of nanoparticles being captured at the test line, leading to a weak fluorescence signal. Obviously, the signal at the test line is inversely proportional to the concentration of CAP in the sample. The detection result could be either resolved with naked eye or quantitatively analyzed by recording the result with a digital camera followed by simple data processing with Adobe Photoshop software (Adobe Systems Inc., San Jose, CA, USA). For the latter, the detection signal was quantified by the relative signal intensity at the test line of a sample over a negative control (I/I_0 %) that can be deduced by Eq. 1:

$$I\Big/I_0\% = (B-B_{ex})\Big/(B_0-B_{ex}) \times 100 \quad (1)$$

where B, B_0, and B_{ex} represent the average brightness in the test line area of a to-be-tested sample, a negative control (i.e., zero concentration of CAP), and a positive control (i.e., excess concentration of CAP, respectively, that can be generated with the help of Adobe Photoshop. By plotting a standard curve of I/I_0 % against concentrations expressed as logarithmic values of the standards, unknown CAP levels in samples can be revealed by comparing their values of I/I_0 % relative to the standard curve. Details for the preparation of CAP-protein conjugates, anti-CAP polyclonal antibodies, and conjugation of antibodies to nanoparticles, fabrication of test strips, detection procedure, and data process are provided in Electronic Supplementary Material.

Results and discussion

We first tested the performance of the LFIA by detecting CAP standard solutions (a series of 2-fold-diluted CAP calibrator solutions) and a negative control. After loading 80 μL of test solution on the sample pad and running at room temperature for 30 min, we visualized the results in a signal-acquisition device equipped with an ultraviolet light and then recorded the images with a digital camera [10]. Figure 2a shows the results. We found that the fluorescence intensity at the test lines decreased as the concentration of CAP increased. The decrease in fluorescence intensity for the standards compared with the negative control could be resolved with naked eye when the concentration of CAP was 0.25 ng/mL or higher, this being the visual limit of quantification. Figure 2b is the calibration curve based on the results shown in Fig. 2a and the aforementioned quantification method. It can be concluded from the calibration curve that (1) The dynamic range of LFIA for CAP covers from 0.06 to 4.0 ng/mL, the coefficient of variation (CV) along the whole

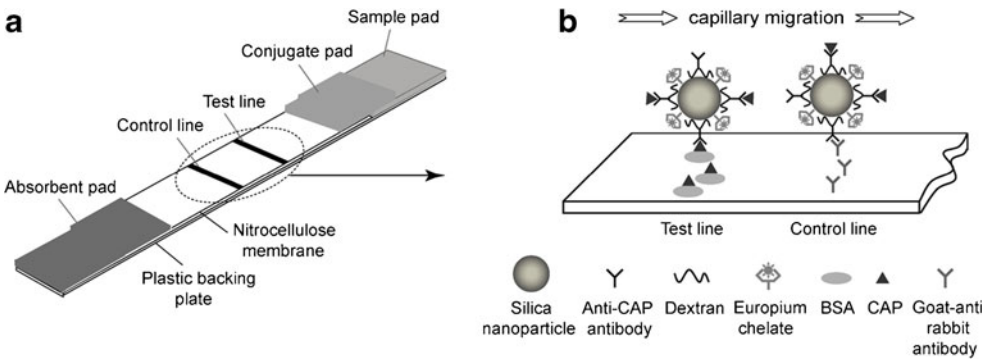

Fig. 1 The principle of Eu(III) nanoparticle-based LFIA of CAP. **a** Scheme showing the components of a test strip; **b** events of immune reactions in the membrane area

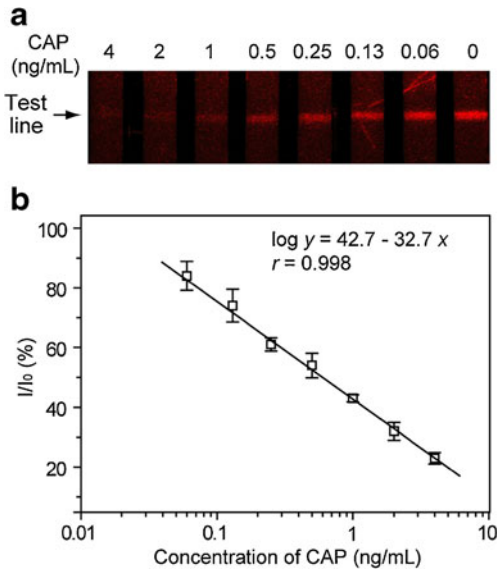

Fig. 2 Eu(III) nanoparticle-based LFIA of CAP standards. **a** Digital image of test strips (membrane area) carried out with 2-fold serially diluted CAP standards and a negative control in assay buffer; **b** calibration line obtained from panel (**a**). At each concentration point, the error bar indicates the SD from six independent measurements

range being less than 10 % (n=6); (2) the half-maximal inhibition concentration (IC50) was 0.45 ng/mL; and (3) The limit of detection (LOD), defined as the concentration corresponding to a signal that is 3 SD above the zero calibrator [12, 13], was calculated to be 0.03 ng/mL (the raw data for the calculation of LOD is provided in Electronic Supplementary Material). Alternatively, the LODs could also be determined to be 0.03 and 0.06 ng/mL when LOD is defined as the concentrations that inhibit 10 % and 20 % of the blank signal, respectively. The limit of quantification (LOQ), defined as the concentration corresponding to a signal that is 10 SD above the zero calibrator [13], was calculated to be 0.42 ng/mL.

In comparison, the LOD and dynamic range of a conventional colloidal gold-based LFIA for CAP spiked tissue standards using monoclonal antibodies were reported as 5.0 ng/g (or ng/mL) and 1~4 ng/g (or ng/mL) [14], respectively, indicating at least 100-fold increase in sensitivity of our Eu (III) nanoparticle-based LFIA. The significant improvement in detection sensitivity can be ascribed to the highly sensitive Eu (III) nanoparticle labels [10, 11]. It is worth mentioning that currently, most commercial colloidal gold-based LFIA kits of CAP have LOD higher than 0.3 ng/g (or ng/mL) (i.e., 0.3 μg/kg as required by the European Union). One exception is the kit from Charm Sciences, Inc. (Lawrence, MA, USA), which claims a LOD of 0.1 ng/g (or ng/mL). However, this assay needs an additional incubation step at 45 °C for 8 min before detection and a special reader for semiquantitative detection.

We also compared our LFIA with enzyme-linked immunosorbent assay (ELISA) of CAP, which is the most widely used method for detection of CAP in foods because of its high analytical sensitivity and capability to screen large number of samples. To this end, we ran parallel experiments by detecting the same standards using a commercial CAP ELISA kit (Euro-Dianostica BV, Arnhem, The Netherlands), which is one of the most frequently used kits in customs of China. The LOD and dynamic range of this CAP ELISA kit were found to be 0.01 ng/mL and 0.025~2.0 ng/mL, respectively (see Figure S1, Electronic Supplementary Material). These results agreed well with the specifications listed in the manual of the kit as well as those reported in the literature [9, 15]. Although this ELISA kit demonstrated a 3-fold lower LOD than our LFIA method, it requires plate washer and reader, and involves several analysis steps, making it less suitable for on-site detection. In contrast, our Eu (III) nanoparticle-based LFIA needs a simple recording device, involves single-step manipulation, and can be used on site.

The potential application of the aforementioned LFIA was demonstrated by detecting cow milk samples, since CAP is totally banned in milk by the Codex Alimentarius Commission. We first detected spiked cow milk samples to test the recovery and reproducibility of our LFIA. To this end, a CAP-free cow milk sample (as determined by the commercial CAP ELISA kit, Euro-Dianostica BV, Arnhem, The Netherlands) was spiked with CAP at 0.1, 0.3, 1.0, and 3.0 ng/mL, covering the entire dynamic range of the LFIA (see Fig. 2b). Then, each sample of a certain spiking level was analyzed using the LFIA in eight independent experiments. Table S1 (Electronic Supplementary Material) summarizes the average recoveries and values of coefficient of variation (CV) for each sample, where the recoveries are in the range of 89.2 %~131.1 % and the CV values are less than 18.4 %. The overall CV value for the spiked sample analysis was slightly higher than that for the standard solution. This high CV can be attributed to the sample defatting procedure

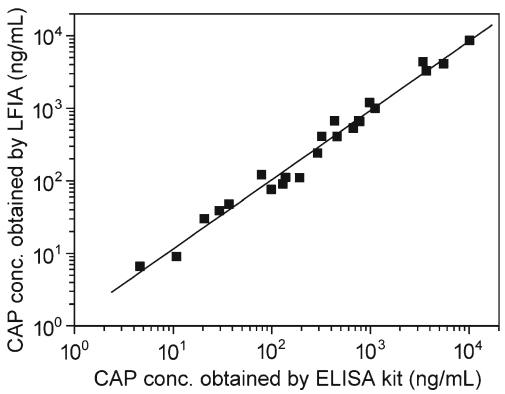

Fig. 3 Correlation between commercial CAP ELISA kit and Eu(III) CAP nanoparticle-based LFIA for quantification of 23 CAP positive cow milk samples (r^2=0.9803, P<0.0001, n=23)

by centrifugation, which may leave residual fat in the solution and, thus, cause interference in CAP detection. The LFIA also showed a good reproducibility as examined by detecting a spiked cow milk sample (1.0 ng/mL) inter-day (CV=17.7, n=6) and intra-day (CV=19.1, n=6). Finally, we correlated our LFIA with the commercial ELISA kit by quantifying 21 cow milk samples that had been previously identified by the commercial ELISA as positive. Because the concentration of CAP in the milk samples may be high enough to exceed the upper limit of the linear range of both methods, these CAP-positive milk samples were diluted with dilution buffer to allow the concentration of CAP to fall into the linear range for each method. The original concentrations of CAP in each milk sample were subsequently calculated by multiplying the measured value by the dilution factor. The obtained correlation coefficient, r^2=0.9803 (n=23), implied good correlation between the two methods and, thus, the possibility of detecting cow milk samples using our LFIA (see Fig. 3).

In summary, we have demonstrated a highly sensitive, competitive lateral flow immunoassay (LFIA) for detection of CAP, of which limit of detections by instrumentation and naked eyes can reach as low as 0.03 and 0.25 ng/mL, respectively. The method was successfully applied to detect cow milk samples with good specificity and sensitivity. It is worth pointing out that our Eu (III) nanoparticle-based LFIA outperforms LFIAs using other labels (e.g., LODs of 0.2 and 5~10 ng/mL were achieved for LFIAs using fluorescent quantum dot [16] and colloidal gold [14] as the labels, respectively), and is comparable to other relatively time-consuming techniques such as ELISA [6, 17] and chemiluminescent ELISA [18] in terms of detection sensitivity. Considering that this new lateral flow immunoassay for CAP is rapid, highly sensitive, and easy to use, we believe it would find applications in the analytical field where trace amounts of CAP need to be monitored or quantified.

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