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Short communication

# Rapid and sensitive detection of β-agonists using a portable fluorescence biosensor based on fluorescent nanosilica and a lateral flow test strip

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ABSTRACT

A portable fluorescence biosensor with rapid and ultrasensitive response for Clenbuterol (CL) has been built up with fluorescent nanosilica and a lateral flow test strip. Quantitative detection of CL was realized by recording the fluorescence intensity of fluorescent nanosilica captured on the test line. The sensing results indicated that the sensitivity of the fluorescent nanosilica-based strip was better than that of conventional colloidal gold-based strips. The visual limit of detection of the strip for qualitative detection was 0.1 ng/mL while the LOD for quantitative detection could down to 0.037 ng/mL by using fluorescence biosensor. The recoveries of test samples were from 89.3% to 97.7%. The assay time for CL detection was less than 8 min, suitable for rapid testing on-site.



## 1. Introduction

Clenbuterol (CL), a representative of the class of synthesized β-adrenergic agonists typically employed as a bronchial, was found to improve growth rate, reduce fat deposition and increase protein accretion (Kuiper et al., 1998; Mitchell and Dunnavan, 1998). However, once the animals are fed with CL, the reside may remain in the meat and liver for a long time as a result of its long half-life, so it may enter the body and distribute throughout the body and result in serious harmful health problems to human such as cardiovascular and central nervous diseases (Martinez-Navarro, 1990). Hence, the use of CL in livestock is banned in many countries including China, the United States, and many European countries (Tan et al., 2009). The main analytical methods for CL detection are enzyme-linked immunoassay (Ren et al., 2009; Zhang et al., 2012; Zheng et al., 2009), liquid chromatography (Freire et al., 2009; Mostafa et al., 2009; Zhang et al., 2011), gas chromatography (Wang et al., 2010; Zhao et al., 2010), electrochemical immunosensors (He et al., 2009; Li et al., 2012), surface plasmon resonance immunosensors (Liu et al., 2011; Wang et al., 2012) and other methods (Kong et al., 2009; Qu et al., 2011). Although these conventional strategies exhibit promising results for sensitive detection of CL, there are still some hindrances including incubation and washing steps before analysis, expensive instrument, long operation times, and tedious sample preparation. The lateral flow test strip (LFTS), also called a dry-reagent strip biosensor has been well-established diagnostic tool in laboratory. This technology offers additional advantages when compared to the conventional detection methods: rapid, simple and cost-effective. The most widely used format of LFTS is the employment of colloidal gold as reporters for colorimetric detection, which was either qualitative or semiquantitative and generally utilized for analyzing residues with relatively high concentrations. In order to meet the requirement of sensitive detection, more labels such as latex (Takanashi et al., 2008), quantum dots (Yang et al., 2011), and up-converting phosphorus technology (Qu et al., 2009) have been employed in LFTS development, but no marked improvement in performance has been achieved. Both the colloidal gold and quantum dots based LFTS have been applied to the detection of

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CL with the limit of detection (LOD) by the naked eye 1 ng/mL and 20 ng/mL, respectively (Zhang et al., 2006; Luo et al., 2011). Some uncertain factors led to this undesirable LOD of quantum dots based LFTS. Consequently, a novel label with immense brightness and ideal photostability is highly desirable for FLFTS applications in CL analysis with ultralow concentrations.

During the past decades, lanthanide chelates have been receiving increasing attention because of their applications as luminescent probes for highly sensitive time-resolved fluoroimmunoassay (TR-FIA), fluorescence microscopy bioimaging, and other analytical techniques (Deng et al., 2010; Oh et al., 2011). They have specific luminescent properties that conventional organic dyes and quantum dots do not have, such as large Stokes shifts, sharp emission profiles, very high levels of brightness, excellent stability against photobleaching and long luminescence lifetimes. The β-diketonate chelate 4,4′-bis(1″,1″,1″,2″,2″,3″,3″-heptafluoro-4″,6″-hexanedione-6″-yl) chlorosulfo-*o*-terphen -yl (BHHCT)–Eu (III) is one of the lanthanide labels that has been proven to give high sensitivity in time resolved fluoroimmunoassay (Xu and Li, 2007; Yuan et al., 1999).

In this work, we report, for the first time, on the development of an optical based biosensors of competitive format which employ a fluorescent nanosilica labeled antibody probe as a reporter for the onsite detection of CL residue in urine samples. We introduced Eu(III)–BHHCT into porous silica nanoparticles to form the stable fluorescent nanosilica with desirable luminescence properties. Due to the advantages derived from lanthanide chelates and LFTS, a rapid, sensitive, specificity, and one-step strategy has been developed for CL analysis. Experimental results demonstrate that fluorescent nanosilica-based LFTS has an excellent ability for quantitative analysis of trace amounts of CL.

## 2. Material and methods

### 2.1. Reagents and materials

Clenbuterol hydrochloride (CL, ≥95%), β-diketonate chelate 4,4′-bis (1″,1″,1″,2″,2″, 3″,3″-heptafluoro-4″,6″-hexanedione-6″-yl)-chlorosulfo-*o*-terphenyl (BHHCT, ≥90%), Tetraethyl orthosilicate (TEOS, 99.999%), (3-Aminopropyl) trimethoxysilane (APTMS, 97%), Europium(III) chloride hexahydrate ($EuCl_3{\cdot}6H_2O$, 99.9%) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Dextran (Mr 500,000) was purchased from Fluka. Staphylococcal Protein-A (SPA, ≥98%) was purchased from ProSpec. The nitrocellulose membrane (SHF-1200420), glass fiber (GF-CP20300), and absorbent paper (C048) was purchased from Millipore. The monoclonal antibody (McAb) against CL was produced in the Key Laboratory of Animal Immunology of the Chinese Ministry of Agriculture.

### 2.2. Preparation of fluorescent nanosilica

Bare silica nanoparticles were synthesized by a reverse microemulsion method. Amino groups were introduced to the surfaces of these particles by treatment with (3-aminopropyl) trimethoxylsilane (APTMS) (Wang et al., 2008). The aminated nanoparticles were characterized by fourier infrared spectrometer and transmission electron microscopy (TEM) (Fig. S1 and S2) and used for coating with Eu(III)–BHHCT. Typically, 1 mL of 1 mg/mL BHHCT solution was added to 1 mL of 30 mg/mL aminated silica nanoparticles suspension and stirring for 2 h. The nanoparticles were washed with ethanol and resuspended in 1 mL of 0.05 M Tris–HCl buffer (pH 7.8), followed by adding 50 μL of 0.04 M $EuCl_3$. After stirring for 30 min, the nanoparticles were washed and resuspended in 1 mL of ethanol and treated sequentially with TEOS, APTMS, BHHCT and $EuCl_3$ as above for another four times. The final product was resuspended in ethanol and stored at 4 °C before use. The fluorescence spectrum of the Eu(III)–BHHCT-coated silica nanoparticles was obtained using a Cary Eclipse fluorescence spectrophotometer (Fig. S3).

### 2.3. Preparation of fluorescent nanosilica-anti-CL McAb probe

A Eu(III)–BHHCT-coated silica nanoparticles-anti-CL McAb conjugate was prepared by an improved periodate oxidation method. Briefly, 250 mg of dextran was added to 5 mL of 0.5 M freshly prepared $NaIO_4$ solution to react overnight; the oxidized dextran was dialyzed against water, then mixed with 5 mg of fluorescent nanosilica suspended in 50 μL, 25 mM carbonate buffer (CBS, pH 9.5). After shaking for 3 h, the nanoparticles were washed and resuspended in 50 μL of CBS and then mixed with 100 μL of optimal anti-CL McAb. After a 12 h reaction, 150 μL of 0.5 M $NaBH_3CN$ was added and the mixture was shaken for another 8 h at 4 °C. The antibody-linked nanoparticles were blocked (10 mM Tris–HCl buffer containing 2% BSA, 4% sucrose, and 1% glycine, pH 7.8), rinsed by centrifugation twice and stored at 4 °C.

### 2.4. Fabrication of fluorescent nanosilica-based FLFTS

The fluorescent nanosilica-based FLFTS is composed of a sample application pad, conjugation pad, nitrocellulose membrane, absorption pad, and a backing card as shown in Fig. 1A. Both the sample pad (15 mm × 30 cm) and conjugation pad (7 mm × 30 cm) were made from glass fiber. The conjugation pad was prepared by dispensing a desired volume of fluorescent nanosilica labeled McAb onto the glass fiber pad using an XYZ Biostrip Dispenser, followed by drying at room temperature before stored at 4 °C. The nitrocellulose membrane (2 × 30 cm) was spotted using the same dispenser with the optimal CL–BSA and SPA applied in the test and control lines, leaving a 0.5 cm space

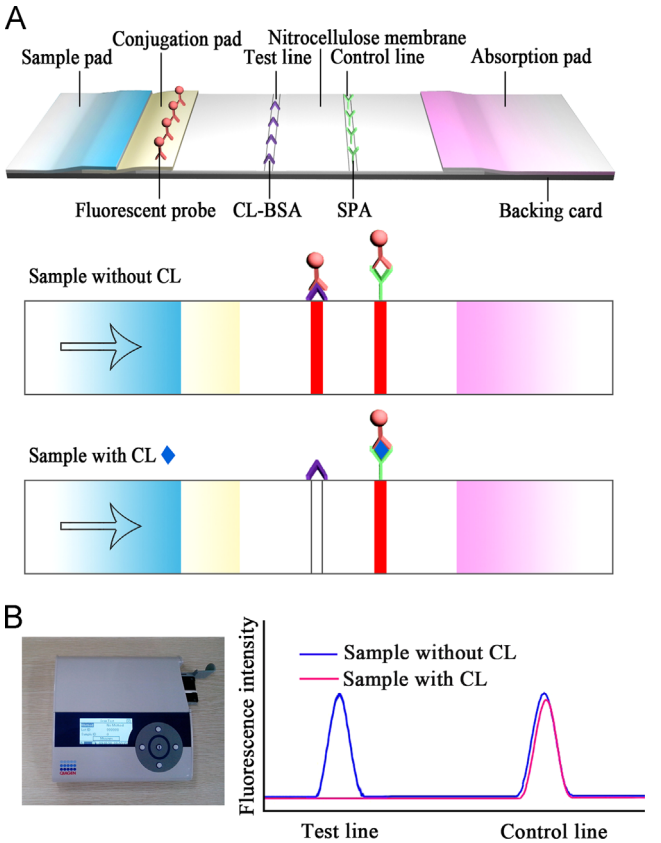


**Fig.1.** (A) Schematic diagram of the principle for the detection of fluorescent nanosilica-based strip and (B) the test strip reader and the typically results.

between the two lines. After drying for 1 h at 40 °C, the membrane was blocked with 2% (w/v) BSA and then dried, sealed, and stored under dry conditions. The sample pad, conjugate pad, blotted membrane, and absorption pad were assembled on the plastic backing support board (4 × 30 cm) sequentially with a 1–2 mm overlap. The master card was cut into 3 mm wide strips using a CM 4000 Cutter (Bio-Dot). The strips were then sealed in a plastic bag with desiccant gel and stored at 4 °C.

### 2.5. Detection of the CL

The detection of CL was carried out by applying 80 μL appropriate standard test CL solution to the bottom of the strip. 5 min later, the results of test line were observed by eye under UV light or the intensity of luminosity on the test line was determined by the ESE-Quant Lateral Flow Reader (Qiagen, Germany) (Fig. 1B).

### 2.6. Detection of CL in swine urine samples

Negative swine urine samples provided by the Supervision and Verification Center of the Ministry of Agriculture, Zhengzhou, China, were previously authenticated by GC–MS and used as the real samples. For each test of CL in swine urine samples, the samples were first centrifuged at 4000 g for 10 min at RT and the supernatants collected. Spiked samples were prepared by adding CL at specified concentrations. The spiked samples and nonspiked samples were all analyzed three times. The results were further confirmed by ELISA.

## 3. Results and discussion

### 3.1. Principle and results judgments

The principle of the designed fluorescent nanosilica-based strip method as shown in Fig. 1A was based on the competitive reaction between the CL–BSA (test line) and the target CL to combine with fluorescent nanosilica-anti-CL McAb probe. When CL is present in the sample, it will bind to the antibody probe, diminishing the binding of said probe to CL–BSA at the test line causing its luminosity intensity to become weaker. Regardless of the presence of target CL in the detection solution, SPA in the control line will combine with IgG Fc fragment of the anti-CL McAb ensuring the validity of the detection. To measure the sensitivity of the developed method, the visual detection limit of the strip was defined as the minimum target concentration producing the luminosity at the test line significantly different from the test line of a negative control strip run without target analyte. The limit of detection (LOD) for quantitative detection was defined as the concentration of CL in the sample solution that caused a 10% decrease of the luminosity at the test line compared with that produced by the negative control.

### 3.2. Parameter Optimization

The amount of fluorescent nanosilica-anti-CL McAb probe loaded on the glass fiber could directly influence the final luminosity of the test and control lines. Stock probe solution was diluted to different concentrations for further testing. The luminosity results of 10-fold, 20-fold and 30-fold dilution were compared and a 20-fold dilution of probe solution was found to be optimal for detection.

The optimum concentration of anti-CL McAb for fluorescent nanosilica labeling was found to be 1 mg/mL, e.g. 1:12.8 dilutions from the stock concentration of McAb at 12.8 mg/mL.

The optimum solute composition for the fluorescent nanosilica-anti-CL McAb probe buffer solution were determined by trial and error, and found to be: 0.01 M Tris–HCl buffer, containing 2 g/L BSA, 1 g/L $NaN_3$, 9 g/L NaCl.

The appropriate concentrations of CL–BSA and SPA applied to NC membrane were 0.4 mg/mL and 0.8 mg/mL.

### 3.3. Sensitivity and stability of the detection

Based on the above optimized detection conditions, CL standard solutions at different concentrations were analyzed using the fluorescent nanosilica-based strip. Each sample was examined in triplicate and typical results are shown in Fig. 2. From these results, we found that the intensity of the red luminosity at the control line was near constant for all strips, validating strip performance. As expected as the concentration of CL in the sample increased, the luminosity at the test lines was reduced. Furthermore, the intensity of red luminosity on test line at 0.1 ng/mL of CL was significantly weaker than that at zero concentration. Thus, 0.1 ng/mL of CL was considered to be the visual LOD for the strip and these detection results were quantified by the test strip reader. The fluorescence intensity on test line was recorded and plotted as a function of various concentrations of CL. Fig. 3A shows the typical response of this biosensor for CL with different concentrations. As can be seen from the figure, well-defined peaks were observed and the peak area decreased along with the increasing of target concentration while no obvious fluorescence signal for the sample of 1.6 ng/mL could be detected. A quantitative calibration curve of the detection was constructed by drawing the logarithmic values of scanned peak area values against the logarithmic concentrations of CL. The relationship between the target concentration and corresponding Peak-Area is shown in Fig. 3B and the LOD for the quantitative detection was calculated as 0.037 ng/mL. This LOD of the fluorescent nanosilica-based strip was better than that of the colloidal gold-based strip method and comparable to that of the ELISA method.

The stability of the strip was examined by using the same batch of strips at 0.2 ng/mL of CL after storage for 0 day, 2 days, 2, 4, 8, 12 and 16 weeks at 4 °C. The visible red luminosity on the test line performed using 16 week old strips was exactly the same as that stored for 0 day. The fluorescent nanosilica-based strips after 16 weeks storage at 4 °C were undoubtedly still usable.

### 3.4. Cross-reactivity of the test strip

To establish the specificity of the test strip, swine urine samples were doped with CL and competitors. The competitors including ractopamine, salbutamol, cimaterol, penicillin, sulfadiazine, chloramphenicol, neomycin, tetracycline, olaquindox, and enrofloxacin were presented at the concentration of 1000 ng/mL. The luminosity of test line was the same as that of the negative control sample. We conclude that the fluorescent nanosilica-based strips have no cross-reactivity with other likely veterinary drugs.

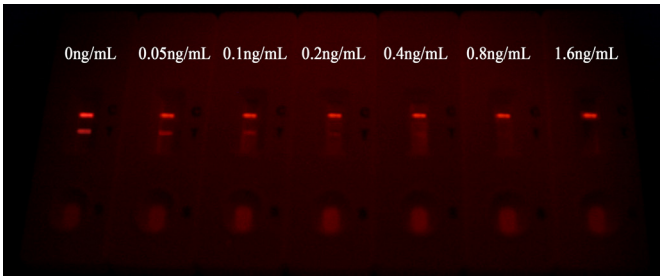


**Fig. 2.** Detection of CL by fluorescent nanosilica-based strips.

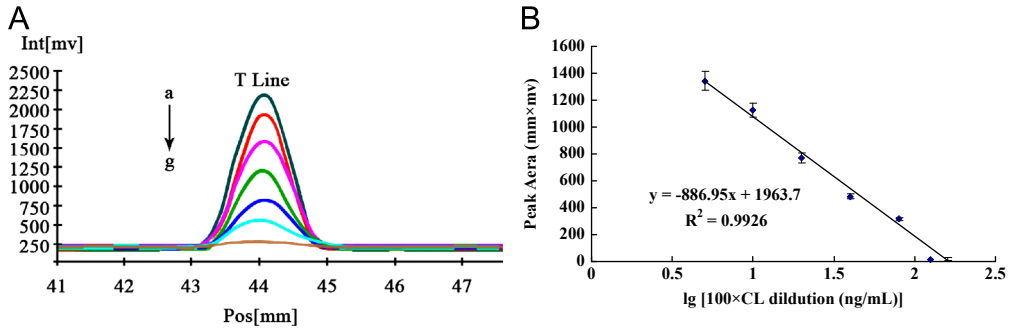


**Fig. 3.** (A) Fluorescence biosensor response for 0 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.2 ng/mL, 0.4 ng/mL, 0.8 ng/mL, and 1.6 ng/mL CL (curves a–g), respectively and (B) fluorescent nanosilica-based strip detection curve of CL by the test strip reader.

### *3.5. Sample analysis*

In order to test the practical applicability and accuracy of the fluorescent nanosilica-based strip method, CL in the swine urine samples was detected. Simulated samples were prepared by doping swine urine samples with known amounts of CL. The average determined recoveries were used as the evaluation index. As shown in Table S1, recoveries were from 89.3% to 97.7%, which clearly meets analytical requirements.

## 4. Conclusions

Fluorescent nanosilica loaded with Eu(III)–BHHCT as a promising alternative reporter was successfully integrated with lateral flow tests strip and first developed for rapid, sensitive, and one-step quantitative detection of a trace amount of CL. This portable biosensor takes advantage of the speed and low cost of conventional immunochromatographic strip as well as high sensitivity and photostability of fluorescent nanosilica-based fluorescent immunoassay. Under optimal conditions, the LOD for the qualitative detection by visual inspection of the strip was as low as 0.1 ng/mL while the LOD of the quantitative procedure is 0.037 ng/mL, which was much better than the colloidal gold-based strip. Overall, the fluorescent nanosilica-based strip could be a potential alternative format of the immunochromatographic test strip for rapid and sensitive on-site screening and detection of CL as well as for other hazardous substances in clinical diagnostics, basic discovery, screening applications and a variety of other biomedical applications.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.06.022.

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