Dual-mode immunoassay system based on glucose oxidase-triggered Fenton reaction for qualitative and quantitative detection of danofloxacin in milk

Suhua Wang, Bolong Fang, Meifang Yuan, Zexiang Wang, Juan Peng, and Weihua Lai*

State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China

ABSTRACT

In this study, a novel colorimetric and fluorescent dual-mode ELISA based on glucose oxidase (GOx)-triggered Fenton reaction was developed for the qualitative and quantitative detection of danofloxacin (DAN). In this system, streptavidin-linked biotinylated anti-DAN-monoclonal antibody (SA-Bio-mAb) and biotinylated GOx (Bio-GOx) form the immune complex mAb-Bio-SA-Bio-GOx. In the absence of DAN, the mAb-Bio-SA-Bio-GOx would be immobilized by combining with coated DAN-BSA and catalyzed glucose to generate H_2O_2 . The Fenton reaction between H_2O_2 and Fe^{2+} generated hydroxyl radicals, which oxidized the α -phenylenediamine to 2,3-diamino-phenazine. A dual-signal immunoassay with colorimetry and fluorescence as the signal readout was established. In the presence of DAN, DAN and DAN-BSA competed with Bio-mAb, decreasing the connection between immune complexes and DAN-BSA and finally resulting in lower signal of colorimetry and fluorescence. Under optimal conditions, the limit of detection of the fluorescence immunoassay was 0.337 ng/mL and was 5.24-fold lower than that of traditional ELISA. The colorimetric immunoassay cut-off value was 30 ng/mL in milk. The average recoveries of the method for milk samples that are spiked with different concentrations of DAN were 91.1 to 128.3%, with a coefficient of variation of 0.7 to 8.2%. These results of the method exhibited good agreement with those of liquid chromatography-tandem mass spectrometry system (LC-MS/MS) method. In brief, this work provides an improved screening strategy with high sensitivity and accuracy for the qualitative or quantitative detection of DAN in milk monitoring.

Key words: colorimetry and fluorescence, ELISA, qualitative and quantitative detection, danofloxacin

INTRODUCTION

Danofloxacin (DAN) is a third-generation animal-specific fluoroquinolone with wide antibacterial spectrum and strong bactericidal activity (Aliabadi and Lees, 2003). It has high concentration in animal lung tissue and good antibacterial effect on respiratory pathogens (Tian et al., 2019). According to Announcement No. 235, Maximum Veterinary Drug Residue in Animal Food, of the Ministry of Agriculture of China (2002), the maximum residue limit of DAN in milk was set at 30 ng/mL.

In milk, DAN has been detected by various analytical methods, including liquid chromatography (LC) with fluorescence detection (Zhang et al., 2017), LC-tandem mass spectrometry (LC-MS/MS; Moreno-González et al., 2014). However, these apparatus methods require expensive equipment, complicated pretreatment processes, and skillful operators (Wen et al., 2012).

A rapid, convenient, and low-cost method for qualitative and quantitative detection of DAN needs to be established. The most popular technique is ELISA, due to its high throughput and low cost (Rissin et al., 2010). However, the traditional ELISA has a low sensitivity (Sheng et al., 2009). Recently, glucose oxidase (GOx), which catalyzes glucose to produce H_2O_2 , has been widely used in biosensors (Leskovac et al., 2005; Xiong et al., 2018). The mixture of $Fe(II)$ - H_2O_2 is regarded as the Fenton reagent. This mixture has remarkable oxidation capability, and it is widely used in environmental chemistry for degrading organic pollutants of wastewater (Boulangé et al., 2019). More research has shown that the oxidative effect of the Fenton reagent is heavily related to the hydroxyl radicals ($\bullet OH$) referred as intermediated (Lacasa et al., 2019). Recently, colorimetric assay of $\bullet OH$ based on the formation of electroactivity or optical active substrate induced by Fenton reaction has been reported (Jiao et al., 2017; Lai et al., 2017; Peng et al., 2019). The Fenton reaction progressed with accompanying occurrence of enzyme catalyst reaction, and then the obtained $\bullet OH$ effectively initiated the catalytic oxidation of α -phenylenediamine

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*Corresponding author: talktolaiwh@163.com

(OPD) to 2,3-aminoazobenzene (DAP), resulting in the production of colorimetric and fluorescence signals.

In the present work, an ELISA method was proposed using GOx to oxidize glucose to produce H_2O_2 , and then Fe^{2+} was added to generate $\bullet OH$, which can accelerate the self-oxidation from OPD to DAP with the change of colorimetry and fluorescence. Colorimetric and fluorescence signals are used for qualitative and quantitative detection of DAN, respectively. The proposed method may provide an improved screening strategy with high sensitivity and accuracy.

MATERIALS AND METHODS

Materials and Equipment

Bovine serum albumin, Tween 20, streptavidin (SA), D-(+)-glucose, biotin-3-sulfo-N-hydroxy-succinimide ester sodium salt, and GOx were from Sigma-Aldrich Chemical Co. (St. Louis, MO). We purchased OPD from Aladdin Biochemical Technology Co. Ltd. (Shanghai, China) and acquired DAN, ofloxacin (OFL), lomefloxacin (LOM), gatifloxacin (GAT), cinoxacin (CIN), fleroxacin (FLR), ciprofloxacin (CIP), enrofloxacin (ENR), marbofloxacin (MAR), sarafloxacin (SAR), sulfamethazine (SMZ), and olaquinox (OLA) from Yuan Ye Biotechnology Co. Ltd. (Shanghai, China). The 96-well ELISA microplates were bought from ExCell Bio (Shanghai, China). Black 96-well microplates were purchased from Corning Inc. (Corning, NY). We purchased DAN-monoclonal antibody (mAb) from Wuxi ZodoBoer Biotech Co. Ltd. (Wuxi, China). Horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin was purchased from Shanghai Universal Biotech Co. Ltd. (Shanghai, China). Negative milk samples, confirmed by LC-MS/MS, and 20 real milk samples were provided by Jiangxi Provincial Veterinary Medicine Feed Supervision Institute (Nanchang, Jiangxi, China). All other chemicals and reagents were of analytical grade and were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). The LC-MS/MS system was composed of a triple-quadrupole instrument (Agilent 6410) and an LC system (Agilent 1200 series; Agilent Technologies, Lexington, MA). A Varioskan LUX multimode microplate reader was purchased from Thermo Fisher Scientific Co. (Waltham, MA) to measure the fluorescence signal. The commercialized ELISA kit was purchased from Reagen Co. Ltd. (Moorestown, NJ).

Preparation of Bio-mAb and Bio-GOx

The Bio-mAb and Bio-GOx were synthesized as described previously with some modifications (Xiong

et al., 2018). For Bio-mAb preparation, 1 mL of mAb (1 mg/mL) was added into 750 μL of biotin-3-sulfo-N-hydroxysuccinimide ester sodium salt (1 mg/mL) and 250 μL of PBS (0.01 M, pH 7.4) and then stirred at room temperature for 3 h. The reacting mixture was dialyzed in PBS (0.01 M, pH 7.4) for 72 h. For Bio-GOx preparation, 6.5 mL of GOx (4.38 mg/mL) was added to 600 μL of biotin-3-sulfo-N-hydroxysuccinimide ester sodium salt (1.7 mg/mL) and stirred at room temperature for 2 h. The reacting mixture was dialyzed in PBS (0.01 M, pH 7.4) for 72 h.

Proposed ELISA Procedure

Coated antigen and Bio-mAb concentrations were first optimized using a chessboard design. The coated antigen was diluted into 2, 1.5, 1, 0.75, 0.5, 0.25, and 0.1 $\mu g/mL$ in carbonate buffer solution (0.01 M, pH 9.6). Then, 100 μL of the diluted solution was added to ELISA wells and incubated at 37°C for 2 h. The ELISA wells were washed with a PBS and Tween solution (PBST; 0.01 M PBS containing 0.05% Tween 20, vol/vol) 3 times. The ELISA wells were blocked with 260 μL of blocking buffer (0.01 M carbonate buffer solution containing 0.2% gelatin) and then incubated at 4°C overnight. After the ELISA wells were washed with PBST 3 times, 50 μL of standard solution and 50 μL of Bio-mAb (2, 1.5, 1, 0.75, 0.5, 0.25, and 0.1 $\mu g/mL$) were sequentially added to each well. After being incubated at 37°C for 30 min, the ELISA wells were washed with PBST 3 times, and then 100 μL of SA was added to each well, and they were incubated at 37°C for 30 min. After the plates were washed with PBST 3 times, 100 μL of Bio-GOx was added, and they were then incubated at 37°C for 30 min. After the plates were washed with PBST 3 times, 100 μL of glucose dissolved in PBS was added to each well, and they were incubated at 37°C for 1 h. Thereafter, 50 μL of Fe^{2+} and 50 μL of OPD were added to the ELISA wells. The ELISA wells were then read at 487 nm for the optical density value (OD_{487}) and at 569 nm for the fluorescence intensity (excited wavelength was 417 nm).

Optimization of Proposed ELISA

To obtain optimal parameters, the study analyzed the different concentrations of the following: SA (0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5 $\mu g/mL$), Bio-GOx (0.05, 0.1, 0.5, 1.0, and 2.0 $\mu g/mL$), glucose (0, 1, 5, 10, 25, and 50 mM), Fe^{2+} (0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 25 mM), and OPD (0, 1, 2.5, 5, 10, 25, and 50 mM), as well as the pH value of the Fenton system (0.5, 1, 2, 3, 4, and 5).

Detection of DAN in PBS

The DAN standard solutions were prepared by diluting the DAN stock solution with PBS (0.01 M, pH 7.4) to different DAN concentrations (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL). Each DAN standard solution was determined three times by the proposed ELISA. The response curve was established by plotting fluorescence signal intensity against the concentration of DAN.

Specificity of Proposed ELISA

The specificity of our proposed method was evaluated by analyzing DAN, DAN structural analogs (OFL, LOM, GAT, ENR, MAR, SAR, CIN, FLR, and CIP), SMZ, and OLA. All drug concentrations were 10 ng/mL, and all experiments were repeated 3 times.

LC-MS/MS Analysis

The proposed ELISA for detection of DAN was further confirmed by a triple-quadrupole LC-MS/MS system (Agilent Technologies). The sample was pre-treated according to Chinese National Standard, 2008 (GB/T22985-2008). Milk samples (2.0 g) and 10 mL of acetonitrile were added to a 50-mL centrifuge tube and shaken with a vortex mixer for 1 min. After centrifugation at $11,040 \times g$ for 5 min at room temperature, the supernatant was transferred to another centrifuge tube and evaporated at 50°C. Five milliliters of PBS (0.05 M, pH 3) was added to the centrifuge tube, shaken with a vortex mixer for 1 min, purified by solid-phase extraction column, and then filtered with a 0.22- μ m cellulose membrane. The LC-MS/MS system was controlled by Mass Hunter software (Agilent Technologies). The chromatographic separation was performed with an Agilent Zorbax Eclipse XDB C₁₈ column (150 mm $\times$ 2.1 mm, 5 μ m) maintained at 30°C. The mobile phase consisted of solvent A (0.1% acetic acid water) and solvent B (methanol). The initial solvent gradient condition was set at 80%. The linear gradient decreased by 40% from 0 to 5.0 min, and then remained unchanged from 5.0 min to 9.0 min. Starting at 9.10 min, the gradient was set to rebalance the column under initial conditions to 11.0 min. The flow rate was 0.20 mL/min, and the injection volume was 15 μ L in full circulation. Ionization was achieved using electrospray ionization in positive ion mode. Detection was conducted in multiple reaction monitoring mode and dissolved with high-purity nitrogen, cone gas, and collision gas. The monitoring ion pairs were chosen as DAN m/z 358.14/340.00 (quantitative ion) and 358.14/339.83 (qualitative ions).

Detection of Milk

The negative samples of milk confirmed by LC-MS/MS were spiked with DAN at 10, 20, 30, and 40 ng/mL for qualitative analysis and at 2.5, 5, 10, and 25 ng/mL for quantitative analysis. The accuracy and precision of the proposed ELISA were estimated by calculating their recovery ratio and coefficient of variation (CV) in quantitative analysis. Each measurement was performed thrice. Twenty real milk samples were determined using the proposed ELISA and HPLC-MS/MS.

RESULTS AND DISCUSSION

Principle of the Proposed ELISA

Figure 1 illustrates the proposed ELISA. In the absence of DAN, Bio-mAb was captured by DAN-BSA that is immobilized on the microplates. After addition of SA and Bio-GOx, the mAb-Bio-SA-Bio-GOx complex was formed. Produced by the oxidation of glucose under the catalysis of GOx, H₂O₂ reacts with Fe²⁺ to produce $\bullet$ OH. The $\bullet$ OH then oxidizes OPD to DAP, with a yellow color and fluorescence. In the presence of DAN, GOx was not immobilized on the microplates and could not produce H₂O₂. The OPD could not be oxidized to DAP with colorimetric and fluorescent signals.

Analysis of Optical Characterization in OPD and DAP

Figure 2 shows an optical characterization of OPD and DAP. The UV-visible absorption peak of DAP was 487 nm. However, OPD did not have a UV-visible absorption peak (Figure 2A). The maximum fluorescent excitation and maximum fluorescent emission wavelength of DAP were 417 nm and 569 nm, respectively. However, OPD did not have fluorescent excitation and emission peaks (Figure 2B). A large stoke shift (>150 nm) is evident for DAP and can effectively eliminate the interference of various nonspecific fluorescence, leading to high sensitivity. The absorption intensity and fluorescence intensity of DAP are significantly enhanced compared with those of OPD. Moreover, the oxide (DAP) of OPD had a significant colorimetric change. Colorimetry and fluorescence of DAP could be used as signal outputs for the proposed method.

Optimization of Key Parameters

The concentrations of coated antigen and Bio-mAb were optimized using a chessboard method. When the concentration of coated antigen and Bio-mAb were

Figure 1. Schematic of detection of danofloxacin (DAN) by a dual-mode signal output system based on glucose oxidase (GOx)-triggered Fenton reaction ELISA. mAb = monoclonal antibody; SA = streptavidin; OPD = *o*-phenylenediamine; DAP = 2,3-aminoazobenzene.

1 and 0.75 $\mu\text{g/mL}$, the fluorescence intensity of the DAN-negative sample was higher than those of others (Supplemental Table S1, <https://doi.org/10.3168/jds.2020-18256>). Therefore, the optimum concentrations of coated antigen and Bio-mAb were selected at 1 and 0.75 $\mu\text{g/mL}$, respectively. Figure 3A shows the effect of SA concentration on fluorescence intensity. When the concentration of SA was increased from 0.05 $\mu\text{g/mL}$ to 1.0 $\mu\text{g/mL}$, the fluorescence intensity gradually increased from 0.32 arbitrary units (a.u.) to 5.38 a.u.. As the concentration of SA was increased to 5 $\mu\text{g/mL}$, the fluorescence intensity remained steady at approximately 5.38 a.u. Thus, the optimal concentration

of SA was 1.0 $\mu\text{g/mL}$. Figure 3B shows the effect of the concentration of Bio-GOx on fluorescent intensity. When the concentration of Bio-GOx was increased from 0.05 $\mu\text{g/mL}$ to 0.5 $\mu\text{g/mL}$, the fluorescence intensity gradually rose to 5.53 a.u. When the concentration of Bio-GOx was increased to 2 $\mu\text{g/mL}$, the fluorescence intensity continued at approximately 5.53 a.u. To obtain a higher fluorescence intensity, we selected 0.5 $\mu\text{g/mL}$ as the optimal concentration of Bio-GOx. Figure 3C shows the effect of the concentration of glucose on fluorescence intensity. The fluorescence intensity increased from 0.07 to 5.20 a.u. when glucose concentration was increased from 1 to 25 mM. When the

Figure 2. Optical characterization of 2,3-aminoazobenzene (DAP) and *o*-phenylenediamine (OPD). (A) UV-visible absorption spectrum of DAP and OPD. (B) Excitation (Ex) and emission (Em) spectra of DAP and OPD. a.u. = arbitrary units.

concentration of glucose was increased to 50 mM, the fluorescence intensity remained at approximately 5.20 a.u. Thus, the optimal concentration of glucose was 25 mM. Figure 3D shows the effect of the concentration of Fe^{2+} on fluorescence intensity. The concentration of Fe^{2+} was increased from 0.01 to 10.0 mM, and the fluorescence intensity gradually increased to 5.00 a.u. As the concentration of Fe^{2+} was increased to 25 mM, the fluorescence intensity remained stable. Therefore, the optimal concentration of Fe^{2+} was 10 mM. Figure 3E shows the effect of pH on fluorescence intensity. When the pH value was in the range of 0.5 to 1.0, the fluorescence intensity remained stable at approximately 5.68 a.u. Subsequently, as the pH was increased to 5, the fluorescence intensity decreased to 0.9 a.u. Therefore, the optimal pH was 1. These results implied that a strong acid pH was beneficial for achieving a higher fluorescence intensity for DAP. However, the pH was lower than that reported in most studies (Mijangos et al., 2006; Abbas et al., 2010). The reason might be that the OPD was easy to oxidize, and we controlled the response time within 5 min. The strong acid (pH 1) was beneficial for achieving a higher fluorescence intensity for DAP in a short time. Thus, the optimal pH was selected at 1. Figure 3F shows the effect of OPD concentration on fluorescence intensity. When the concentration of OPD was increased from 1 to 10 mM, the fluorescence intensity gradually increased to 5 a.u. With further increase of OPD concentration from 10 to

50 mM, fluorescence intensity remained stable. Therefore, the optimal concentration of OPD was 10 mM.

Standard Curve for DAN of Proposed ELISA

The standard curves of the proposed ELISA based on colorimetric and fluorescent intensity in PBS were established. Figure 4 shows the calibration curve constructed by plotting the fluorescence intensity against the logarithm of DAN concentration (0 to 100 ng/mL). The linear equation of the proposed method for quantitative detection of DAN can be described as $Y = -2.756 \lg X + 4.480$ ($R^2 = 0.997$), with a good linear detection range from 1 ng/mL to 25 ng/mL. In the equation, Y is the fluorescence intensity and X is the DAN concentration. The limit of detection (LOD), defined as the blank signal minus 3 standard deviations (Guo et al., 2016), was calculated as 0.337 ng/mL, indicating that LOD of the fluorescence immunoassay was 5.24-fold lower than that of conventional ELISA (Supplemental Figure S1, <https://doi.org/10.3168/jds.2020-18256>). Supplemental Figure S2 (<https://doi.org/10.3168/jds.2020-18256>) shows that the linear equation based on colorimetric signal for quantitative detection of DAN was $Y = -0.603 \lg X + 0.786$ ($R^2 = 0.993$), with a good linear detection range from 1 to 10 ng/mL. The LOD was 0.684 ng/mL. Supplemental Table S2 (<https://doi.org/10.3168/jds.2020-18256>) exhibits the LOD of this method and others. This result indicated that the

Figure 3. Parameter optimization in proposed ELISA. (A) Streptavidin concentration (SA, 0.05–5 µg/mL); (B) biotinylated glucose oxidase concentration (Bio-GOx, 0.05–2 µg/mL); (C) glucose concentration (0–50 mM); (D) Fe^{2+} concentration (0.01–25 mM); (E) pH (0.5–5); and (F) α -phenylenediamine concentration (OPD, 0–50 mM). Error bars indicate SD ($n = 3$).

Figure 4. Calibration curve of proposed fluorescence ELISA. The danofloxacin (DAN) concentration ranges from 0 to 100 ng/mL. The inset shows a linear range of 1 to 25 ng/mL. LOD = limit of detection. Error bars = SD.

LOD of the proposed method was lower than that of the other methods listed in Supplemental Table S2 and could be used for quantitative detection of DAN. The LOD of our proposed ELISA was more sensitive than that of the commercialized ELISA kit (Supplemental Figure S3, <https://doi.org/10.3168/jds.2020-18256>). When OPD was added as enzyme substrate in the commercialized ELISA kit, the LOD was higher than that of the proposed ELISA (Supplemental Figure S4, <https://doi.org/10.3168/jds.2020-18256>).

Specificity of Proposed ELISA

The specificity of this method was evaluated by analyzing DAN, DAN structural analogs (OFL, LOM, GAT, ENR, MAR, SAR, CIN, FLR, and CIP), SMZ, and OLA. All drug concentrations were 10 ng/mL. Figure 5 shows that the fluorescence intensity of other drugs was approximately 5 a.u., whereas the fluorescence intensity of DAN was 1.58 a.u. This result indicates that the proposed ELISA has good specificity.

Accuracy and Precision of Proposed ELISA

Figure 6 shows the negative samples of milk confirmed by LC-MS/MS spiked with DAN at 10, 20, 30, and 40 ng/mL for qualitative analysis. The cut-off value was 30 ng/mL by naked-eye detection. The accuracy and precision of the proposed ELISA were further compared with those of the LC-MS/MS method by detecting 4 DAN-spiked milk samples. The recovery of fluorescence ELISA was 95.3 to 125% with CV of 1.9 to 8.2%. The recovery of HPLC-MS/MS was 73.1 to 118%, with CV of 0.7 to 6.3% (Table 1). These results indicate an acceptable accuracy and precision of the proposed ELISA for the quantitative detection of DAN in actual milk samples. Supplemental Table S3 (<https://doi.org/10.3168/jds.2020-18256>) shows that 1 real milk sample was positive (sample 6) and 19 real milk samples were negative for presence of DAN using the proposed ELISA and LC-MS/MS. The detection result of the ELISA was compatible with that of the HPLC-MS/MS method.

Figure 5. Specificity of this method in detecting danofloxacin (DAN) with 9 other quinolones [ofloxacin (OFL), lomefloxacin (LOM), gatifloxacin (GAT), enrofloxacin (ENR), marbofloxacin (MAR), sarafloxacin (SAR), cinoxacin (CIN), fleroxacin (FLR), and ciprofloxacin (CIP)], sulfamethazine (SMZ), and olaquinox (OLA). a.u. = arbitrary units. Error bars indicate SD ($n = 3$).

CONCLUSIONS

In this study, a novel colorimetric and fluorescent dual-mode immunoassay system was developed based on GOx-triggered Fenton reaction for qualitative and quantitative detection of DAN. The cut-off value was 30 ng/mL by naked-eye detection. The proposed fluorescent ELISA demonstrates a high sensitivity for DAN detection, with LOD of 0.337 ng/mL, which is ap-

proximately 5.24-fold lower than that of a conventional ELISA. The average recoveries for milk samples spiked with different concentrations of DAN ranged from 91.1 to 128%, with a CV that ranged from 0.7 to 8.2%. The accuracy and precision of the proposed method was further confirmed using the LC-MS/MS method. The proposed ELISA method provides an improved screening strategy with high sensitivity for the qualitative or quantitative detection of DAN in milk monitoring.

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Figure 6. Colorimetric immunoassay in milk at different danofloxacin (DAN)-spiked concentrations. (A) 10 ng/mL, (B) 20 ng/mL, (C) 30 ng/mL, and (D) 40 ng/mL.

Table 1. Comparison of addition and recovery of fluorescence ELISA and liquid chromatography-tandem mass spectrometry (LC-MS/MS) detection¹

DAN added (ng/mL)	Fluorescence ELISA (n = 3)			LC-MS/MS (n = 3)		
	DAN (ng/mL)	Recovery (%)	CV (%)	DAN (ng/mL)	Recovery (%)	CV (%)
2.5	2.445	97.8	5.7	2.114	84.6	6.3
5	6.233	125	8.2	5.924	118	4.6
10	9.529	95.3	5.4	9.210	92.1	0.7
25	24.869	99.5	1.9	18.275	73.1	1.3

¹DAN = danofloxacin.

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ORCIDS

Suhua Wang <https://orcid.org/0000-0002-4554-5923>