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Universal simultaneous multiplex ELISA of small molecules in milk based on dual luciferases

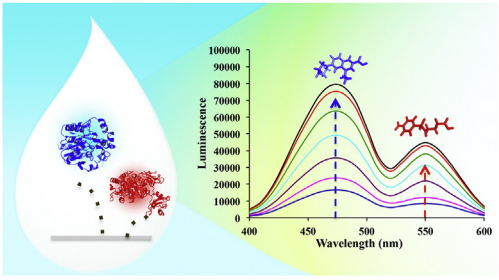
Xuezhi Yu [a, 1], Xiya Zhang [a, 1], Zhanhui Wang [a], Haiyang Jiang [a], Ziquan Lv [b], Jianzhong Shen [a], Guoliang Xia [c], Kai Wen [a, *]

[a] College of Veterinary Medicine, China Agricultural University, Beijing Key Laboratory of Detection Technology for Animal-Derived Food Safety, Beijing Laboratory for Food Quality and Safety, No.2 Yuanmingyuan West Road, Haidian District, Beijing 100193, People's Republic of China
[b] Department of Genetic Toxicology, Shenzhen Center for Disease Control and Prevention, No.8, Longyuan Road, Longzhu Road, Nanshan District, Shenzhen 518020, People's Republic of China
[c] State Key Laboratory for Agro-Biotechnology, College of Biological Science, China Agricultural University, No.2 Yuanmingyuan West Road, Haidian District, Beijing 100193, People's Republic of China

HIGHLIGHTS

- It realized the really synchronous detection with simple signal generation and collection procedure.
- It could simultaneously detect 20 fluoroquinolones and 21 sulfonamides residues in milk with simple dilution below the maximum residue limit.
- It was even more sensitive than the chemiluminescence immunoassays.

GRAPHICAL ABSTRACT



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ABSTRACT

The enzyme-linked immunosorbent assay (ELISA) has become the most important and widely used rapid detection technology for food safety because of its simple operation, fast speed and high sensitivity. Multiplex synchronous detection is the goal of ELISA that is always pursuing for. However, the reported multiplex ELISAs have not truly realized synchronous detection because of the complex signal generation and collection procedures. Here, we developed a dual-luciferases competitive direct bioluminescent immunoassay (DBL-cdELISA) with only one substrate addition step followed immediately by simultaneous signal acquisition. It is the first report of simultaneous multiplex analysis of small molecules based on microtiter plates and enzymes without any additional steps. The IC_{50} values for norfloxacin (NOR) and sulfamethazine (SMZ) were 0.051 ng mL^{-1} and 0.211 ng mL^{-1}, respectively. The results demonstrated that the application of different luciferases and substrates simplified the signal generation and collection procedures and enabled simultaneous detection of small molecules with a simple procedure, high throughput and fast speed, that will be of great significance for the development of multiple assays.

1. Introduction

The enzyme-linked immunosorbent assay (ELISA) has become the most important and widely used rapid detection technology for

* Corresponding author.
E-mail address: wenkai@cau.edu.cn (K. Wen).
[1] These authors contributed equally to this paper.

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food safety due to its simple operation, fast speed and high sensitivity [1–4]. Multiplex synchronous detection is the goal of ELISA that is always pursuing for [5,6]. The multiplex immunoassay (MIA), which can detect two or more chemical classes simultaneously has attracted increasing attention due to its high detection efficiency, short assay time, low sample consumption, and minimal overall cost [7–9]. For this reason, various multiplex immunochemical techniques, including ELISA, chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) [8], lateral flow immunoassays [10,11], microarrays [12,13] and flow-through immunoaffinity chromatography test [14] have been developed. While, the key to multiplex ELISA is the separation of signals from different analytes. To achieve this goal, many multiplex immunoassays placed immunoreagents specific to different analytes in separate wells within the same test system [1,15].

In order to further simplify thees assays, multiplex immunoassays have been developed that can be performed in the same well. In studies by Porstmann. T and Jiang. W′, horseradish peroxidase (HRP) and alkaline phosphatase (ALP) were employed to produce signals from two different targets. The ALP substrate was first added and the plate was washed before the HRP substrate was added to the same wells [5,16]. In other examples of chemiluminescence ELISAs, the chemiluminescent signals were recorded at different time points with no intervening washing steps after the two substrates were added to the same wells [7,8]. However, the additional signal generation and collection procedures were somewhat complex. Therefore, the purpose of our study was to develop a simultaneous multiplex ELISA that would enable us to record separate signals simultaneously after only one addition of the appropriate substrates.

Luciferases have become important research tools over the last two decades due to their ability to emit light (bioluminescence, BL) by the oxidation of substrate. The two main classes of luciferases employed as research tools are the firefly luciferase (Fluc) and Renilla luciferase (Rluc) [17–20]. Fluc is adenosine triphosphate (ATP)-dependent, uses D-luciferin as its substrate, and emits light at about 560 nm, whereas Rluc is ATP-independent, uses coelenterazine as its substrate, and emits BL at about 480 nm [21,22]. Because the substrates of Fluc and Rluc are different and do not interfere with each other, and because the difference between emission wavelengths is 80 nm, the dual-luciferase system has been used in many areas, including gene expression, in vivo imaging and protein-protein interactions [23–26]. However, to the best of our knowledge, there has been no report focusing on the application of dual-luciferase systems in immunoassays of multiple low-molecular-weight chemical residues.

Herein, we utilized fluoroquinolones (FQs) [27] and sulfonamides (SAs) [28] as model molecules to develop a dual-luciferases competitive direct bioluminescent immunoassay (DBL-cdELISA) for the simultaneous screening of 20 FQs and 21 SAs residues in milk (see Scheme 1). The method presented here enables real simultaneous detection with high sensitivity and specificity by recording separate signals simultaneously after addition of the appropriate substrates in a single step. We believe that this is the first report of a one-step DBL-cdELISA method for the detection of multiple chemical residues of low molecular weight which has potential for future applications.

2. Material and methods

2.1. Materials

The *anti*-FQs single-chain variable fragment (scFv), scFv-C4A9H1_mut2 [29] (FQs-scFv) and *anti*-SAs scFv-4D11 (SM-scFv) [30] were established previously in our laboratory. Norfloxacin-ovalbumin (OVA-NOR) conjugates and 6-(4-aminophenylsulfonamido) hexanoic acid - OVA (OVA-HS) conjugates were preserved in our laboratory [31,32]. The Rluc and Fluc genes were obtained from Promega (Madison, WI, USA). D-luciferin, coelenterazine-h and isopropyl β-D-1-thiogalactopyranoside (IPTG) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The HisTrap™ HP column was from GE Healthcare (Beijing, China). DAB Horseradish Peroxidase Color Development Kit was purchased from TIANGEN Biotech Co., Ltd. (Beijing, China).

The buffers and solutions were prepared with water purified using a Milli-Q system from EMD Millipore Corporation (Belleria, MA, USA). Norfloxacin (NOR), sulfamethazine (SMZ), other FQs standard solutions, SAs standard solutions and all media used in this study were prepared as described in the literature [29,31]. White opaque high-binding plates were purchased from Costar (Cambridge, MA, USA). Luminescence was measured using a SpectraMax M5 microplate reader from Molecular Devices (Downingtown, PA, USA). All other chemicals and organic solvents were of reagent grade and were obtained from Beijing Chemical Co. (Beijing, China).

2.2. Buffers

Coating buffer was 0.05 M carbonate buffer (CB), pH 9.6. Washing buffer was 0.01 M phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4) containing 0.1% Tween 20. Blocking buffer was 0.01 M PBS containing 5% skim milk. Antibody and fusion protein dilution buffer were both 0.01 M PBS.

2.3. Preparation of FQs-scFv-Rluc and SAs-scFv-Fluc fusion proteins

The genes encoding *anti*-FQs scFv, *anti*-SAs scFv, Rluc and Fluc were amplified and assembled using splicing by overlapping extension-polymerase chain reaction (SOE-PCR) to yield full-length FQs-scFv-Rluc and SAs-scFv-Fluc genes with a reference amino acid linker (GSTSGSGKPGSGEGSTSG) [33] and *Eco*RI and *Xho*I restriction sites. After restriction digestion and ligation reaction, the pET22b (+) vector containing the FQs-scFv-linker-Rluc or SAs-scFv-linker-Fluc was transformed into *E. coli* BL21 (DE3) competent cells, respectively. After identification by polymerase chain reaction (PCR) and DNA sequencing, positive colonies were preserved in medium containing 30% glycerol at −80 °C.

After optimization of the expression conditions, the positive colony was cultivated overnight at 37 °C in 2 × YT medium containing 100 μg mL^{-1} ampicillin (AMP) in a shake flask (200 rpm). The overnight cultures were transferred into 100 mL fresh 2 × YT medium containing 100 μg mL^{-1} AMP and cultivated on the shaker at 200 rpm until the OD_{600nm} of the culture reached approximately 0.6–1.0. IPTG was added at a final concentration of 0.05 mM to induce the expression of the fusion protein at 16 °C, 200 rpm for 16 h. The cells were harvested by centrifugation (5000 × g) and the soluble FQs-scFv-Rluc and SAs-scFv-Fluc proteins were extracted and purified using a 5 mL HisTrap™ HP column according to the manufacturer's instructions. The purified protein was dialyzed against PBS and characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

2.4. Procedure for BL-cdELISA$_{FQs\text{-}scFv\text{-}Rluc}$ and BL-cdELISA$_{SAs\text{-}scFv\text{-}Fluc}$

The white opaque high binding plate was coated with 400 ng/well of OVA-NOR or 300 ng/well OVA-HS in the CB at 37 °C for 2 h. After washing three times and drying, the plates were blocked with 200 μL/well of blocking buffer for 1 h at 37 °C. Following the

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