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Multi-color quantum dot-based fluorescence immunoassay array for simultaneous visual detection of multiple antibiotic residues in milk

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ABSTRACT

Antibiotic residues, which are among the most common contaminants in animal-based food products such as milk, have become a significant public health concern. Here, we combine a multicolor quantum dot (QD)-based immunofluorescence assay and an array analysis method to achieve simultaneous, sensitive and visual detection of streptomycin (SM), tetracycline (TC), and penicillin G (PC-G) in milk. Antibodies (Abs) for SM, TC and PC-G were conjugated to QDs with different emission wavelengths ($QD_{520\ nm}$, $QD_{565\ nm}$ and $QD_{610\ nm}$) to serve as detection probes (QD-Ab). Then a direct competitive fluorescent immunoassay was performed in antigen-coated microtiter plate wells for simultaneous qualitative and quantitative detection of SM, TC, and PC-G residues, based on fluorescence of the QD-Ab probes. The linear ranges for SM, TC and PC-G were 0.01–25 ng/mL, 0.01–25 ng/mL and 0.01–10 ng/mL, respectively, with detection limit of 5 pg/mL for each of them. Based on fluorescence of the QD-Ab probes, residues of the three antibiotics were determined visually and simultaneously. Compared with a commercial enzyme-linked immunosorbent assay kit, our method could achieve simultaneous analysis of multiple target antibiotics in multiple samples in a single run (high-throughput analysis) and improved accuracy and sensitivity for analysis of residues of the three antibiotics in authentic milk samples. This new analytical tool can play an important role in ameliorating the negative impact of the residual antibiotics on human health and the ecosystem.

1. Introduction

Various food safety incidents in recent years have increased the need for food safety monitoring for contaminants such as drug residues and illegal additives. Among various possible contaminants in animal-based food products, antibiotics residues are of particular interest, due to their frequent use in animal husbandry, not only for treatment of diseases but also for prophylactic and prevention purposes and to improve agricultural productivity. Consumption of animal products contaminated with antibiotic residues can cause allergic reactions in humans and reduce the efficacy of antibiotics for treatment human infections. The resulting increase in antibiotic-resistant bacteria is a public health concern. Moreover, residues of antibiotics are the most frequently detected contaminants in milk and other dairy products (Chafer-Pericas et al., 2010). These developments have increased the demand for accurate, sensitive and high-throughput analytical methods for the determination of antibiotic residues in foodstuffs. Several methods for detection of antibiotic residues are currently available, including microbiological assays (Aureli et al., 1996; Lourenco et al., 2013), instrumental assays (Bohm et al., 2012; Wang et al., 2006) and immunoassays (Bang-Ce et al., 2008; Meng and Xi, 2011; Zhao et al., 2007). However, microbiological assays are time consuming and have relatively poor sensitivity and specificity. Instrumental assays require complex sample preparation and expensive equipment. Thus, these approaches cannot fulfill the demand for fast, easy and simultaneous detection of antibiotic residues in milk. Immunoassays can offer higher sensitivity and

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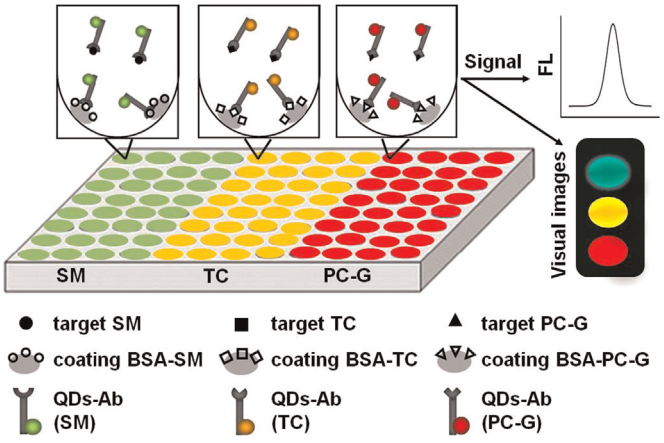
specificity, along with greater convenience. However, the most common enzyme-linked immunosorbent assay (ELISA) and conventional fluorescence immunoassay (FIA) do not provide high-throughput quantification of multiple analytes (e.g. several different antibiotics). Therefore, the development of new methods for the accurate, sensitive, rapid, and convenient detection of multiple antibiotics residues in milk remains an important need in food safety monitoring.

Photoluminescent semiconductor nanocrystals, such as quantum dots (QDs), have excellent fluorescence characteristics (e.g., stable, narrow, and tunable emission spectra with broad excitation spectra) compared with conventional organic fluorescent dyes (Alivisatos, 1996; Bruchez et al., 1998; Medintz et al., 2005). As a result, they have been increasingly used as fluorescent labels in FIA (Esteve-Turrillas and Abad-Fuentes, 2013; Goldman et al., 2002; Hu et al., 2010; Nichkova et al., 2007; Yang and Li, 2006; Zhu et al., 2011) for the detection of pathogens (Wang et al., 2011; Yang and Li, 2006; Zhao et al., 2009), proteins (Cao et al., 2011; Hu et al., 2010; Wang et al., 2007a; Wang and Mountziaris, 2013), and small molecules (Esteve-Turrillas and Abad-Fuentes, 2013; Garcia-Fernandez et al., 2014; Peng et al., 2009; Zhu et al., 2011). Zhu et al. (2011) developed an immunoassay using dual-color QDs and enzymes to achieve simultaneous detection of multiple chemical residues in milk. However, their method provided only qualitative results. Recently, Dzantiev et al. developed an immunochromatographic test for detection of several antibiotics based on multicolor QD, which shows a great potential convenient strategy for daily food safety control without aid of experiment instrument. However, the minimum detected values of visual qualitative analysis in their work is higher than the maximum residue limits (MRLs) required by the EU Commission Regulation (Taranova et al. 2015).

The aim of this work is to achieve simultaneous, sensitive and visual detection of residues of multiple antibiotics in milk by combining multicolor QD-based competitive fluorescence immunoassay (mQD-cFIA) and array analysis. Streptomycin (SM), tetracycline (TC) and penicillin G (PC-G) are the most frequently used among the aminoglycoside, tetracycline, and penicillin families of antibiotics, and are employed to treat a variety of infectious diseases of cows. Thus, they were selected as the model targets in this study. Their structures are shown in Fig. S1. Their corresponding antibodies (Abs) were covalently coupled to QDs with peak emission wavelengths of 520 nm, 565 nm and 610 nm, respectively, to construct the detecting probes (QD-Ab). As shown in Scheme 1, three coating antigens of SM, TC and PC-G were fixed in different areas of a microtiter plate, and the corresponding three kinds of antibiotics were sensitively detected based on cFIA using the QD-Ab probes.



Scheme 1. A schematic illustration of the multi-analyte assay for three kinds of antibiotics by mQD-cFIA.

2. Experimental

2.1. Materials and instruments

Streptomycin sulfate (SM), tetracycline hydrochloride (TC), penicillin G (PC-G), kanamycin (KM), doxycycline (DC), penbritin (PB) and albumin bovine V (BSA) were purchased from Gen-view Scientific, Inc. (USA). Streptomycin-BSA (SM-BSA), tetracycline-BSA (TC-BSA), penicillin G-BSA (PC-G-BSA), anti-SM antibody, anti-TC antibody and anti-PC-G antibody (all are polyclonal antibodies) were purchased from Abcam. ELISA kits for SM, TC and PC-G were purchased from R&D Systems (USA). Thioglycolic acid (TGA), tellurium (Te), cadmium chloride hemipentahydrate ($CdCl_2 \cdot 2.5H_2O$), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC · HCl) and N-Hydroxysuccinimide (NHS) were purchased from Aladdin (Shanghai, China). $NaBH_4$, NaOH, HCl, NaH_2PO_4, Na_2HPO_4, Boric acid, Tris–HCl and ethanol were all of analytical grade. Black polystyrene microtiter plates and black polystyrene microtiter plates with transparent bottom were purchased from Corning Incorporated (USA). Milli-Q purified water (18.2 mΩ, Elga, England) was used in all experiments. Phosphate buffer (PB, containing NaH_2PO_4 and Na_2HPO_4). Phosphate buffer saline (PBS, containing Na_2HPO_4, KH_2PO_4, NaCl and KCl).

The fluorescence spectra were obtained using a fluorescence spectrophotometer (F-7000, Hitachi, Japan). Fluorescence images were obtained under an inverted fluorescence microscope (Olympus IX71, Japan). A microplate reader from Tecan Company (Infinite M200 Pro, Switerland) was used. Photos of electrophoresis gels were taken using a gel imaging system (Vilber Lourmat, France).

2.2. Preparation of QD-Ab detecting probes

The TGA stabilized multicolor CdTe QDs used in this work were synthesized following previous reports (Gaponik et al., 2002; Zhao et al., 2007b). See Supporting information for further details of the synthetic protocol and the spectra of the resulting QDs (Fig. S2). The QDs were conjugated with mAbs for each corresponding antibiotic to produce QD-Ab probes using EDC and NHS as coupling reagents (So et al., 2006; Wang et al., 2007b). To do so, QDs (2 nmol/mL) were first activated with EDC (1.6 mg/mL) and NHS (0.2 mg/mL) for 0.5 h at room temperature, and then mixed with mAbs (4 μg/mL) with shaking for 2 h at room temperature. Finally, the reaction was blocked by centrifugation using a millipore amicon (MW cutoff: 10 kD) ultra-0.5 mL centrifugal filter and the product was stored in a refrigerator at 4 °C.

2.3. Preparation of antigen coated microtiter plate

The three coating antigens (BSA-SM, BSA-TC and BSA-PC-G) were embedded in each well in the different sections of the black microtiter plate (Scheme 1) by the following procedure. Firstly, the microtiter plate was exposed under UV light (254 nm) for 1 h, followed by adding coating antigens (with concentration of 10 μg/mL in 0.01 M PBS, pH 7.4) and incubating overnight at 4 °C. Then excess binding sites were blocked with 5% (w/v) BSA in 0.01 M PBS (pH 7.4) for 2 h at 37 °C after removing excess coating antigens by washing several times with washing buffer (PBS containing 0.05% Tween-20, PBST). The microtiter plate modified with coating antigen was then ready for further use.

2.4. Quantification of three kinds of antibiotics by mQD-cFIA assay

The three model antibiotics (SM, TC and PC-G) were determined by mQD-cFIA as follows, with all measurements done in triplicate. Firstly, 100 μL of standard free target antigens at a series