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METHODS AND PROTOCOLS

Quantitative fluorescent detection of tetracycline in animal-derived foods using quantum dots

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Abstract

Tetracycline (Tc) antibiotics, a class of synthetically produced broad-spectrum antimicrobial drugs, have been widely used in animal husbandry, leading to their widespread presence in animal-derived foods. However, misuse, overuse, and non-compliance with withdrawal periods in animal farming have resulted in excessive Tc residues in these foods, which can cause various adverse reactions in humans, induce bacterial resistance, and pose a significant threat to public health. Consequently, the detection of Tc antibiotic residues in animal-derived food has become a critical issue. This study aims to establish a novel method for quantifying Tc residues in animal-derived food using quantum dots (QDs) fluorescence immunoassay (FLISA). The developed method was optimized to achieve a detection limit of 0.69 ng/mL and a quantitative detection range of 1.30 ~ 59.22 ng/mL. The applicability of the method was demonstrated by successfully determining Tc residues in pork, chicken, fish, milk, eggs, and honey samples spiked with Tc standard solutions, yielding recoveries ranging from 94.01% to 110.19% and relative standard deviations between 1.10% and 11.39%. The significance of this study lies in its potential to provide a rapid and reliable approach for monitoring Tc residues in animal-derived food products, thereby contributing to the enhancement of food safety monitoring practices.

Key points

- *Screen out tetracycline-specific blocking monoclonal antibodies*
- *The quantitative detection has high specificity and sensitivity*
- *This method can be a useful tool for laboratories or testing facilities*

Keywords Tetracycline · Quantum dots · Fluorescence immunoassay · Animal-derived foods

Introduction

Tetracycline (Tc) antibiotics are a class of broad-spectrum antibiotics widely used in the treatment of human diseases and animal husbandry. Due to their effective antibacterial properties, low cost, and minimal side effects, they are also used as drug additives (Sapadin and Fleischmajer 2006). However, the extensive use of Tcs has led to excessive residues in daily foods like meat, milk, eggs, and honey (Oka et al. 2000; Muriuki et al. 2001; Peres et al. 2010; Zhang et al. 2014). Residual Tcs can enter the human body through the food chain, leading to various adverse effects such as poisoning, teratogenicity, carcinogenicity, bacterial resistance, and weakening of the immune system, all of which have a significant impact on human life and health (Martinez 2009; Gary and Fiona 2016; Jansen et al.

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2018). Moreover, due to improper use and the relatively high solubility of Tc in water, Tc pollution in surface water and groundwater has become a serious environmental issue (Guo et al. 2010; Chen et al. 2016).

To protect human health, many countries and organizations have set maximum residue limits (MRL) for Tcs. For example, the Chinese Ministry of Agriculture and Rural Affairs, the Codex Alimentarius Commission (CAC), the European Union (EU), and the U.S. Food and Drug Administration (FDA) have established different MRLs for various drugs in different animal species and tissue sites. China, CAC, and the EU have set an MRL of 100 ng/g for Tc in cow and sheep milk, while the FDA has set it at 300 ng/g. For Tc in different animal muscles, China and CAC have set MRLs at 200 ng/g, the EU at 100 ng/g, and the FDA at 2000 ng/g (Guo and Gai 2011). Therefore, it is crucial to develop sensitive and accurate detection methods for Tc residues in animal-derived foods.

Recent advancements in nanomaterials have shown great potential in improving the sensitivity and selectivity of detection methods for various contaminants, including Tc antibiotics. Quantum dots (QDs), semiconductor nanocrystals with radii smaller than or close to the exciton Bohr radius (Lee et al. 2012), have been widely used in research in fields such as biology and medicine due to their excellent spectral characteristics and photochemical stability (Osinski et al. 2009; Valizadeh et al. 2012; Yang et al. 2022). Compared to other bioluminescent dyes, QDs exhibit broad excitation lines, narrow emission lines, good adaptability after surface coating, excellent photostability, and tunable absorption wavelengths, making them ideal as fluorescent markers (Shen et al. 2007). In comparison to using HRP-labeled antibodies in immunoassays, using QDs for antibody labeling offers many advantages, such as eliminating the need for secondary antibodies, avoiding colorimetric reactions, saving time and costs, and significantly improving sensitivity (Zhang et al. 2017).

Several studies have explored the use of nanomaterials, including QDs, in the detection of Tc antibiotics and other contaminants. Khalilov et al. discussed the future prospects of biomaterials in nanomedicine, highlighting the potential of nanomaterials in improving the sensitivity and selectivity of detection methods (Rovshan Khalilov and Nasibova 2024). Rosic et al. reviewed the role of nanobiomaterials in cancer signaling, cell/gene therapy, and diagnosis, demonstrating the versatility of nanomaterials in various biomedical applications (GvozdenRosic and Omarova 2024). Eftekhari et al. developed a sensitive and selective electrochemical detection method for bisphenol A using SBA-15 like Cu-PMO modified glassy carbon electrode, showcasing the potential of nanomaterials in enhancing the performance of electrochemical sensors (Eftekhari et al. 2021).

To determine Tc residues, many methods have been developed. Moats (2000) used liquid chromatography to determine Tc antibiotics in beef and pork tissues, with recoveries typically in the range of 90%-100% and a quantification limit of 0.05–0.1ppm. Xu et al. (2017) combined liquid–liquid microextraction with high-performance liquid chromatography coupled with an ultraviolet detector (HPLC-UVD) to determine Tc residues in milk samples, with an average recovery rate of 92.38%-107.3%, relative standard deviation (RSD) less than 8.66%, and limit of detection (LOD) of 0.95–3.6 μg/kg. Karageorgou and Tsai established HPLC methods to detect Tcs in milk samples (Tsai et al. 2009; Karageorgou et al. 2014). Gab-Allah et al. (2023) developed an ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) method for the analysis of Tc residues in chicken meat using isotope dilution. They obtained high recovery rates of 97.7%-102.6%, RSDs less than 4%, limit of quantitation (LOQ) lower than 0.2 μg/kg, and high sensitivity. Wang et al. (2018) synthesized a molecularly imprinted polymer for the selective recognition of Tc in powdered milk and subsequently quantified Tc extracted from powdered milk samples using UPLC-MS/MS. Under optimized experimental conditions, Tc had LOD of 0.22–0.32 μg/kg, Intra-batch and inter-batch RSDs were in the ranges of 3.8%-6.9% and 2.8%-7.4%, respectively, and spike recoveries were 84.7%-93.9%. The method was successfully applied to the determination of Tc residues in powdered milk. P. Kowalski (2008) used capillary electrophoresis to determine Tc residues in fish samples, with estimated detection limits for analyzed Tcs of 1.3–1.8 ng/g and quantification limits of 4.3–5.9 ng/g. Zhang (2007) prepared an anti-Tc antibody and developed an indirect competitive enzyme-linked immunosorbent assay to detect Tc residues in milk, with a half-maximal inhibitory concentration (IC50) value of 3.92 μg/mL. The specificity of this detection method was studied by determining the cross-reactivity of the antibody with structurally related compounds such as chlortetracycline (112%) and doxycycline (< 2%). Spike recoveries of Tc in raw milk samples were between 74 and 116%, with intra-batch and inter-batch coefficients of variation less than 14.5% and less than 25.0%, respectively. Han et al. (2006) determined Tc using flow injection and inhibitory chemiluminescence detection with copper as a probe ion. The linear range for Tc was 3.6×10^{-8} to 1.0×10^{-5} mol/L, with a detection limit of 5.0×10^{-9} mol/L. This method is applicable for the determination of Tc in drug formulations and human urine, with recoveries between 95 and 105%.

Among the various methods for detecting Tc residues, HPLC is the most common, offering high sensitivity, good stability, and excellent reproducibility. However, it is susceptible to interference from impurities in the sample matrix, and when detecting multiple Tc analogs, the separation may not be ideal, requiring gradient elution for proper separation. Liquid

chromatography-tandem mass spectrometry (LC–MS/MS) provides higher sensitivity, lower detection and quantification limits, and minimal interference from sample matrix, allowing simultaneous detection of a wider range of Tc analogs. However, it is costlier, and sample purification processes can be more complex. Other methods are generally suitable for screening samples but may not be suitable for precise quantitative detection.

To overcome the limitations of traditional methods and leverage the advantages of nanomaterials, we have developed a direct detection fluorescence immunoassay (FLISA) for rapid quantification of Tcs in animal-derived foods, utilizing the covalent binding of QDs with Tc-specific monoclonal antibodies. This novel approach aims to provide a sensitive, selective, and cost-effective method for monitoring Tc residues in food products, contributingto the enhancement of food safety monitoring practices. The significance of this study lies in its potential to address the growing concern of Tc antibiotic residues in animal-derived foods, which pose a significant threat to public health. By developing a rapid and reliable detection method using QDs-based FLISA, this study contributes to the ongoing efforts to ensure food safety and protect consumer health.

In summary, this study aims to develop a novel quantum dots-based fluorescence-linked immunosorbent assay (QDs-based FLISA) method for the rapid and sensitive detection of tetracycline residues in animal-derived foods. The assay conditions will be optimized to achieve high sensitivity, selectivity, and reproducibility. The developed method will be validated by applying it to the determination of Tc residues in various animal-derived food samples, including pork, chicken, fish, milk, eggs, and honey. The performance of the developed method will be compared with traditional detection methods, highlighting its advantages in terms of sensitivity, selectivity, and cost-effectiveness. The potential of the developed method for wide-ranging applications in food safety monitoring and its significance in protecting public health will be demonstrated.

By achieving these objectives, this study contributes to the advancement of food safety monitoring techniques and provides a valuable tool for the rapid and reliable detection of Tc antibiotic residues in animal-derived foods. The successful application of QDs-based FLISA in this context may also inspire further research on the use of nanomaterials in the development of sensitive and selective detection methods for other contaminants in food and environmental samples.

Materials and methods

Materials and reagents

Carboxyl-functionalized water-soluble QDs (ZnCdSe/ZnS, QDs-COOH) were obtained from Wuhan JiaYuan QDs Technology Development Co., Ltd. (Wuhan, China). Tc and doxycycline were sourced from the China National Institutes for Food and Drug Control (Beijing, China), while 4-dimethylaminopyridine (DAMP), chlortetracycline, oxytetracycline, demeclocycline, minocycline, and tigecycline were all obtained from Beijing Solaibao Technology Co., Ltd. (Beijing, China). Bovine serum albumin (BSA), chicken ovalbumin (OVA), and N-hydroxysuccinimide (NHS) were purchased from Sigma (St. Louis, Missouri, USA). Triethylamine was obtained from Aladdin (Shanghai, China), and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) was sourced from China National Pharmaceutical Group (Beijing, China). Horseradish peroxidase-conjugated goat anti-mouse antibody was purchased from Abbkine (Wuhan, China). Anti-Tc mAb 8F9 was prepared in the Key Laboratory of Immunobiology of Henan Province, Zhengzhou University (Zhengzhou, China). 96-well plates were obtained from Corning Inc. (Corning, USA).

Instruments

The Nanodrop 2000c UV–Vis spectrophotometer was purchased from Thermo Fisher Scientific (Massachusetts, USA). A pH meter was procured from Mettler Toledo (Zurich, Switzerland). A high-speed refrigerated centrifuge was obtained from Eppendorf (Hamburg, Germany). The TP-214 analytical balance was purchased from Denver (Denver, USA). Electrophoresis equipment was sourced from Bio-Rad (California, USA). A desktop constant-temperature oscillator (THD-200) was obtained from Beijing Yatekulong Instrument Technology Co., Ltd. (Beijing, China).The SpectraMax i3X multi-mode microplate reader was purchased from Molecular Devices (Silicon Valley, USA). The gel imaging system was acquired from Bio-Rad (California, USA).

Literature search and selection

A comprehensive literature search was conducted to identify relevant studies on the detection of tetracycline in animal-derived foods using quantum dots. The following electronic databases were searched: PubMed, Web of Science, ScienceDirect, and Google Scholar. The search terms used were: ("tetracycline" OR "antibiotic residues") AND ("quantum dots" OR "fluorescent detection" OR "immunoassay") AND ("food" OR "animal-derived"). The search was limited to articles published in English between January 2000 and December 2023. Additional relevant studies were identified by manually searching the reference lists of the retrieved articles.

The inclusion criteria for the studies were: (1) original research articles; (2) studies focusing on the detection of tetracycline in animal-derived foods; (3) studies utilizing

quantum dots for fluorescent detection; and (4) studies providing sufficient information on the detection method, experimental conditions, and analytical performance. Review articles, conference proceedings, and studies not meeting the inclusion criteria were excluded.

Solution preparation

The buffer solutions used included 0.05 M carbonate-buffered saline (CBS, pH 9.6), 0.01 M phosphate-buffered saline (PBS, pH 7.4), and PBS buffer containing 0.05% Tween-20 (PBST). The blocking solution was prepared by combining 5% skim milk powder with PBST. The color-developing solution was created by mixing pre-prepared A and B solutions in a 1:1 ratio. The A solution contained 3.15 g citric acid, 11.56 g trisodium citrate dehydrate, and 0.08 g nonylphenol in deionized water. This mixture was heated and dissolved, and then 0.5 g urea peroxide was added to it, and it was stored at 4 °C. The B solution contained 1.27 g 4-amino-4-methylmorpholine in 500 mL methanol, followed by the addition of 500 mL isopropanol. After thorough mixing, it was stored in the dark at 4℃. 2mol/L sulfuric acid solution was used as the stop solution.

Artificial antigen preparation and identification

The hydroxyl group on Tc was first modified to a carboxyl group using the succinic anhydride method, and then this carboxyl group was coupled to the amino groups contained on the carrier proteins BSA and OVA using the carbodiimide method to prepare the immunogen Tc-BSA and the encapsulated antigen Tc-OVA (Fig. 1). (Yue et al. 2007; Yu et al. 2008) Their identity and conjugation ratios were assessed using UV scanning and SDS-PAGE mobility shift analysis. (Li et al. 2017).

mAb production and evaluation

In accordance with references, two female Balb/c mice aged 6–8 weeks were immunized with Tc-BSA (Huang et al. 2019; Ma et al. 2021). After four dorsal multipoint injections for immunization (50ug/dose), blood was collected from the tail veins, and the antibody potency was evaluated using indirect competitive enzyme-linked immunosorbent assay (ic-ELISA). The spleen from the mouse showing the best immune response was fused with sp2/0 myeloma cells (ATCC® CRL-1581™, Chen et al. 2020; Liu et al. 2021). The selected cell lines were cultured in vitro, and ascites were prepared, followed by purification through ammonium sulfate precipitation (Liu et al. 1999). The purified antibodies were stored at -20 °C. Antibody titer, affinity, and inhibition were assessed using enzyme-linked immunosorbent assay (ELISA) (Bobrovnik. 2003; Suzuki and Sriwilaijaroen 2021).

QDs-based immunoconjugate preparation

For QDs-labeled fluorescent probes, an EDC-based approach was employed to prepare anti-Tc mAb-QDs conjugates (Yue et al. 2007). A schematic representation of the fluorescent probe consisting of anti-Tc mAb and QDs is shown in Fig. 2. In brief, 5 µL of water-soluble QDs with a maximum emission wavelength of 605 nm (ZnCdSe/ZnS, QDs-COOH, 8 mM) were added to a 1.5 mL brown centrifuge tube, followed by the addition of 15.3 µL EDC (1 mg/mL, dissolved in PBS) to achieve a molar ratio of 1:2000 between the QDs and EDC. The mixture was incubated at 25 °C with shaking for 30 min to activate the water-soluble QDs (Zheng et al. 2022). In the second step, 21.58 µL of anti-Tc mAb (8F9, 1.39 mg/mL, dissolved in PBS) was added to the above mixture to achieve a 1:5 molar ratio between the QDs and the antibody. The above mixture was allowed to react under sealed conditions for 3 h at 25 °C in an oscillating incubator. To block excess unreacted carboxyl sites on the QDs, additional BSA solution (100 µg/mL, 10 µL) was added, and the anti-Tc-mAb-QDs solution was blocked with the BSA solution for 30 min. The synthesized fluorescent probes were stored in the dark at 4°C (Wang et al. 2019; Yemets et al. 2022). Subsequently, considering the quantities of QDs, EDC, and anti-Tc mAb in the probe synthesis, this study optimized these synthesis conditions.

Characterization of QDs and fluorescent probes

As per previous research, the fluorescent probes were characterized and analyzed to confirm the successful conjugation of QDs and anti-Tc mAb. UV–visible absorption spectra were obtained using a Nanodrop 2000c spectrophotometer

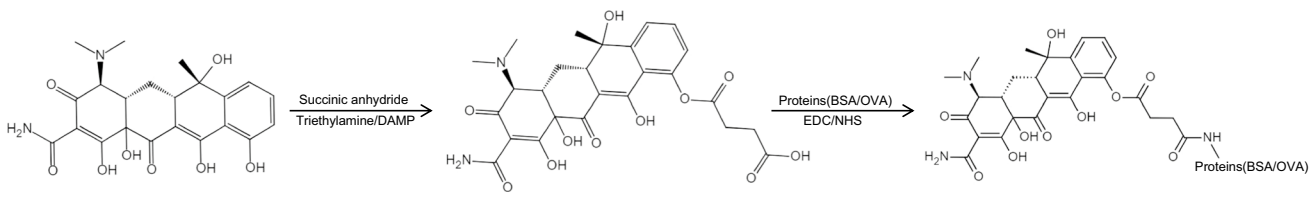

Fig. 1 Schematic representation of the synthesis of Tc-BSA and Tc-OVA complete antigens

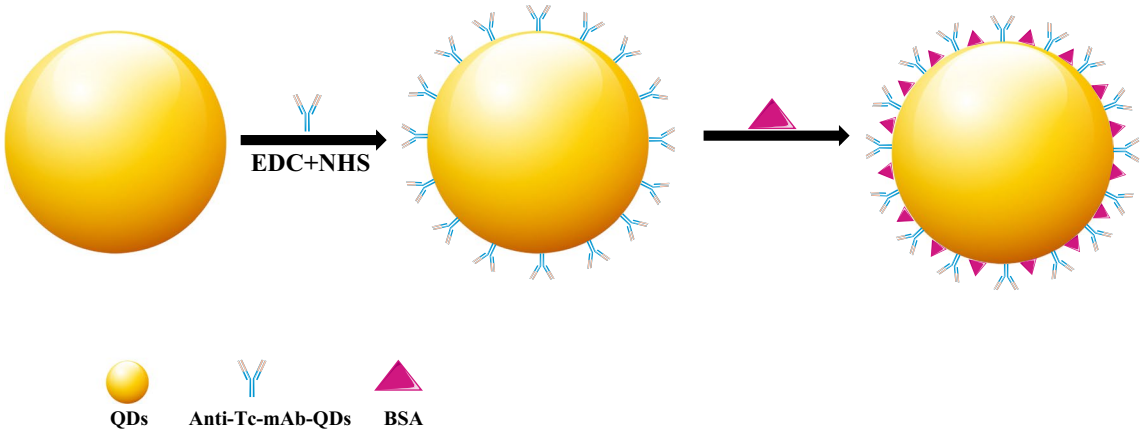

Fig. 2 Schematic representation of the amino group of anti-Tc-mAb coupled to the carboxyl group on the quantum dot

(Guan et al. 2012). The fluorescence spectra of the QD-conjugated anti-Tc mAb and pure QDs were measured using a multi-mode microplate reader. Furthermore, gel electrophoresis, including agarose gel electrophoresis and SDS–polyacrylamide gel electrophoresis (SDS-PAGE), was performed. The conjugation ratio was determined by constructing a standard curve with Tc concentration as the x-axis and its corresponding absorbance values at the maximum absorption peak (355 nm) as the y-axis. Different concentrations of complete antigen corresponded to their respective absorbance values at 355 nm, which were used to calculate the drug concentration in the conjugate in terms of mass concentration (g/L) and then converted to molar concentration (g/mol).

Establishment of FLISA detection method

Following references, a checkerboard method was used to select four different coating antigen concentrations (0.5, 1, 2, 4 μg/mL) and varying dilutions of QD-conjugated antibody (50, 100, 200) to create inhibition curves (Deng et al. 2018; Huang et al. 2019). The IC50 value was determined to identify the optimal coating antigen concentration and QD-conjugated antibody dilution. Subsequently, single-factor experiments were conducted to optimize parameters including antigen coating time, blocking time, dilution of Tc and antibody solution (PBS, PBST), standard competition reaction time (20, 30, 40, 60 min), and probe incubation time. The best reaction conditions should exhibit suitable fluorescence values (FV), a lower IC50, and a higher FV/IC50 ratio. A higher FV/IC50 indicates greater sensitivity. The optimal reaction conditions were used to establish a standard FLISA curve for Tc. In this curve, the vertical axis represents the fluorescence value (F/F_0), F_0 represents the fluorescence value without added Tc, and the horizontal axis represents the logarithm of the Tc concentration.

Specificity of fluorescence immunoassay

Tc-related compounds, including chlortetracycline, oxytetracycline, minocycline, tigecycline, demeclocycline, and doxycycline, were selected as competitors. Direct competitive FLISA was used to determine the IC50 for each competitor against the anti-Tc mAb. The following formula was used to calculate cross-reaction rate (CR): CR = (IC50 for Tc / IC50 for each competitor) × 100%. lower CR indicates higher specificity of the prepared mAb (Bentley et al. 1994; Wang et al. 2009; Subbarayal et al. 2013).

Stability testing

Precision and stability are important parameters for evaluating the reliability and applicability of immunoassay kits. For precision evaluation, FLISA was performed on coated plates at different time points. Additionally, a stability test was conducted by storing the test kit at room temperature. The test was performed at 0, 7, 14, and 28 days, with standard curves constructed at each time point to assess method stability. IC50, FV, and IC20—IC80 were calculated for different batches of plates and various storage times.

Sample processing and method validation

The sample treatment method is based on liquid–liquid extraction with ethylene diamine tetraacetic acid (EDTA) and methanol, followed by a freezing step to facilitate phase separation at low temperatures. After degreasing with hexane, the sample extract was evaporated and resuspended. 5 g of homogenized sample was weighed into a 50 mL centrifuge tube, and 0.1 M aqueous EDTA (7 mL) and methanol (16 mL) were added sequentially, and after shaking and mixing for 5 min, the tube was allowed to stand at -20℃ for 60 min, and then immediately centrifuged for 10 min at 4000g.

8 mL of the supernatant was transferred to another 50 mL centrifuge tube and defatted by adding 8 mL of n-hexane and shaking well for 5 min. The whole mixture was then centrifuged (4000g, 1min). The lower phase (3 mL) was transferred to a 5 mL EP tube and blown dry under nitrogen, and the residue was resuspended with 500 uL methanol (Desmarchelier et al. 2018). For the evaluation of FLISA's analytical efficiency, blank samples were spiked with Tc standard solutions at concentrations of 50, 75, 150, and 300 µg/kg. Following the sample processing method described above, the FLISA and HPLC–MS/MS method was used to determine Tc concentrations in the samples and calculate the recovery rate.

Results

Preparation and identification of artificial antigens

Carrier proteins (BSA, OVA) and Tc exhibit distinct characteristic absorption peaks under UV scanning. After successful conjugation of the Tc to the carrier protein, there are changes in peak shape or position due to the formation of covalent bonds and electron transfer between molecules, allowing for the determination of successful coupling of artificial antigens (Cooper and Paterson 2001). The UV absorption spectra show distinct absorption peaks at 355 nm for the conjugates Tc-BSA and Tc-OVA (Fig. 3a, b), Through the SDS-PAGE results showed that Tc-BSA and Tc-OVA showed significant hysteresis compared to both BSA and OVA (Fig. 3c, d), indicating successful conjugation. Additionally, since Tc drugs are typically yellow, the presence of a noticeable yellow color in the conjugates can also be an indicator of successful coupling, and within a certain range, the higher the reaction ratio, the darker the immunogen color. Furthermore, the standard curve for Tc obtained by UV–visible spectrophotometry is expressed as $y = 0.015X + 0.18$ ($R^2 = 0.93$) (Fig. S1), and the calculated coupling ratios for artificial antigens Tc-BSA and Tc-OVA are 4.36:1 and 2.38:1, respectively.

Antibody titer and inhibition of monoclonal antibodies

After the fourth immunization, the antibody titers and IC50 values of the mouse sera were determined (Cooper and Paterson 2001, Zhou et al. 2012). The results indicate that, at antigen coating concentrations of 2 µg/mL and Tc drug concentrations of 2µg/mL, the antibody titers/IC50 values for the mouse sera in parallel experiments were 204.8 k/439.64

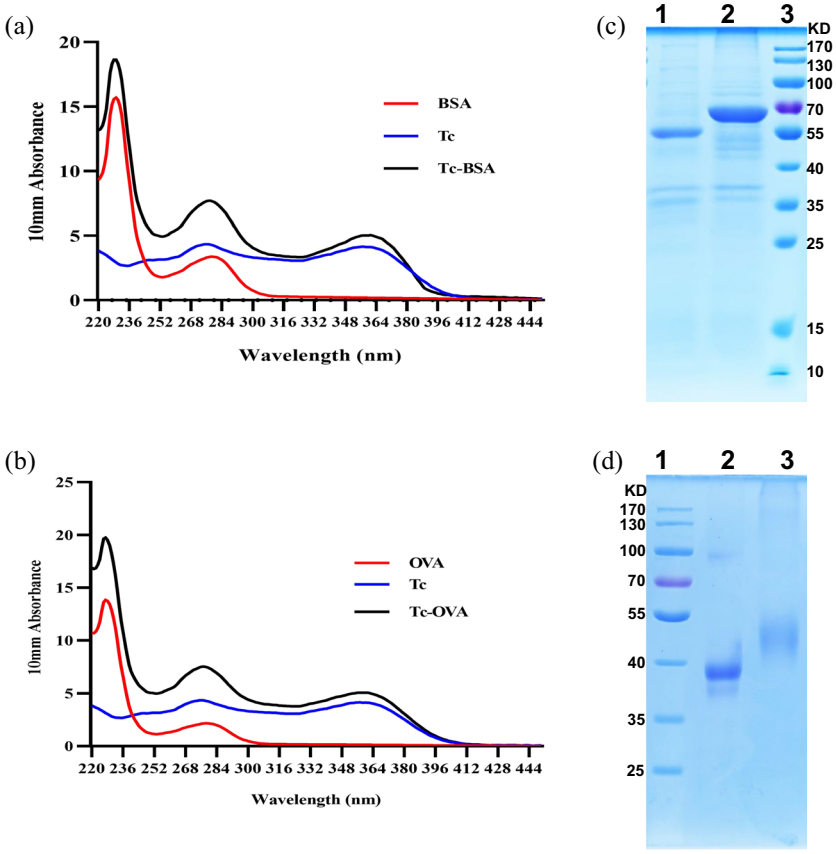

Fig. 3 Identification of Tc-BSA and Tc-OVA complete antigens. (**a**) Spectral analysis for Tc-BSA using UV absorption spectroscopy. (**b**) UV absorption spectroscopy analysis for Tc-OVA. (**c**) SDS-PAGE analysis of Tc-BSA: Lane 1 represents the protein BSA, Lane 2 is Tc-BSA, and Lane 3 is marker. (**d**) SDS-PAGE analysis of Tc-OVA: Lane 1 is the protein marker, Lane 2 is OVA, and Lane 3 is Tc-OVA

ng/mL and 102.4 k/450.68 ng/mL, respectively (Fig. 4). Higher titers and inhibition rates suggest better affinity and sensitivity of the antibodies. Subsequently, mouse #1 was selected for cell fusion. Through ELISA screening (Fig. S2), a hybridoma producing cell supernatant with titers/IC50 of 102.4 k/42.30 ng/mL was obtained (Fig. S3). This cell line was further utilized for cell culture, ascites preparation, and purification to obtain anti-Tc mAb for subsequent experiments (Fig. S4).

Evaluation of fluorescent probe QDs

The results illustrate the fluorescence spectra of anti-Tc-mAb-QDs and pure QDs. The maximum emission wavelength of the QDs is 605 nm, while that of anti-Tc-mAb-QDs is 620 nm (Fig. 5a). As described previously by Song et al. (Oka et al. 2000), the bioconjugation results in an increase in hydrodynamic size of QD-antibody compared to the initial QDs. This redshift may be attributed to the quantum size effect, as the surface of QDs is modified by antibodies, reducing the probability of non-radiative transitions. The coupling process may also enhance dipole–dipole interactions and increase the Stokes shift of QDs. The full width at half maximum (fwhm) of the emission peak for antibody-labeled QDs showed no significant change compared to the QDs. This observation suggests that the coupling process did not cause aggregation or irregular distribution of QDs. Furthermore, gel electrophoresis and SDS-PAGE results, as shown in Fig. 5b and c, demonstrate that QDs migrated faster under the same conditions than anti-Tc-mAb-QDs, indicating that the molecular weight of the fluorescent probe anti-Tc-mAb-QD conjugate is significantly larger than that of the QDs. All data indicate that anti-Tc-mAb-QDs have been successfully prepared. Moreover, the binding between QDs and antibodies forms a covalent bond rather than electrostatic adsorption. The antibody-QD conjugate is stable and can be stored at 4℃ for one month without significant loss of activity (Guan et al. 2012).

Optimization of FLISA conditions and establishment of detection methods

The concentration of immobilized antigen is crucial for improving FLISA sensitivity. The results indicate that when the antigen coating concentration is 2 μg/mL and the probe is diluted 100-fold, the fluorescence reaches saturation (Table S1). Therefore, the antigen coating concentration was set at 2 μg/mL, and the probe was diluted 100-fold. The antigen coating time, blocking time, Tc and antibody dilution solutions (PBS, PBST), standard competition reaction time (20, 30, 40, 60 min), and probe incubation time used during the preparation of standard solutions and samples can influence the sensitivity and precision of FLISA. By studying various conditions, it was found that the best sensitivity was achieved at 37℃ for 2 h of coating, 37℃ for 1 h of blocking, Tc diluted with PBS, and antibody dilution solution with PBST, with a standard competition reaction time of 40 min. Therefore, these conditions were chosen for the assay. Based

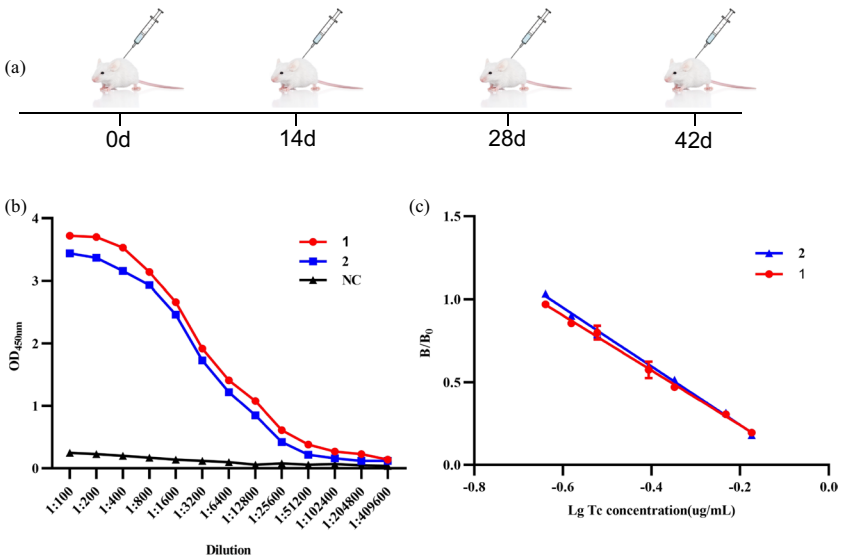

Fig. 4 Immunization procedures and evaluation of immunization in mice. (**a**) Tc-BSA was injected subcutaneously at multiple points on the back, with a total of four immunizations, each two weeks apart. (**b**) Following the four immunizations, mouse No. 1 exhibited a serum potency of 204.8K, while mouse No. 2 displayed a serum potency of 102.4K. (**c**) After undergoing four immunizations, mouse No. 1 exhibited a serum sensitivity IC50 of 439.64 ng/mL with a fitted curve described by the equation y = -1.66X—0.09(R^2 = 0.99). Similarly, mouse No. 2 displayed a serum sensitivity IC50 of 450.68 ng/mL with a regression model represented by the equation y = -1.77X—0.11($R.^2$ = 0.99)

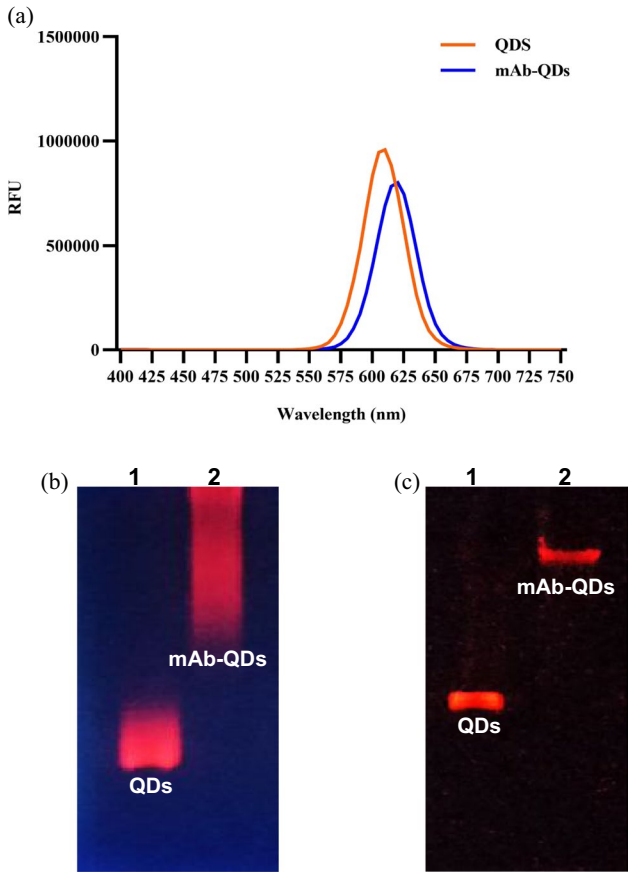

Fig. 5 Characterisation and analysis of fluorescent probes. (**a**) Fluorescence emission spectra of QDs and QDs-labeled antibodies. (**b**) Agarose gel electrophoresis: lane 1 indicates QDs, and lane 2 indicates anti-Tc-mAb-QDs. (**c**) SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis): lane 1 represents QDs, and lane 2 represents anti-Tc-mAb-QDs

on the optimized detection conditions, a standard curve for Tc in FLISA was established. The linear regression equation was y = -0.36X—0.24 (R^2 = 0.99) (Fig. 6a and b). The LOD and IC50 were found to be 0.69 ng/mL and 8.77 ng/mL, respectively, with a linear quantification range of 1.30–59.22 ng/mL (IC20 ~ IC80). Compared to previously established indirect competitive ELISAs (with an LOD of 2.02 ng/mL and IC50 of 9.88 ng/mL), the LOD for this FLISA was at least three times lower (Table 1). Furthermore, when compared to a previous IC50 for Tc of 3.92 µg/mL and a linear detection range (IC20 ~ IC80) of 0.1 ~ 100 µg/mL, with a LOD of 10 ng/mL in an icELISA, this FLISA's LOD is at least 15 times lower. Moreover, the LOD for this method is much lower than the values required for Tc detection in EU and Chinese food regulations (0.1–2 µg/g).

Specificity evaluation of fluorescence immunoassay

The CR of the antibody with analogs can assess the specificity of the antibody. A lower CR indicates higher specificity for the target drug. The FLISA method established in this study used Tc as a reference with a CR of 100%, a CR of 76.43% with chlortetracycline and 2.26% with oxytetracycline, and almost no cross-reactivity with other structurally and functionally similar compounds, such as minocycline, tigecycline, demeclocycline, and doxycycline, indicating excellent specificity for Tc (Table 2).

Elimination of matrix interference effects

Samples were processed using appropriate dilutions of PBS, and the Tc standard curve was determined to assess

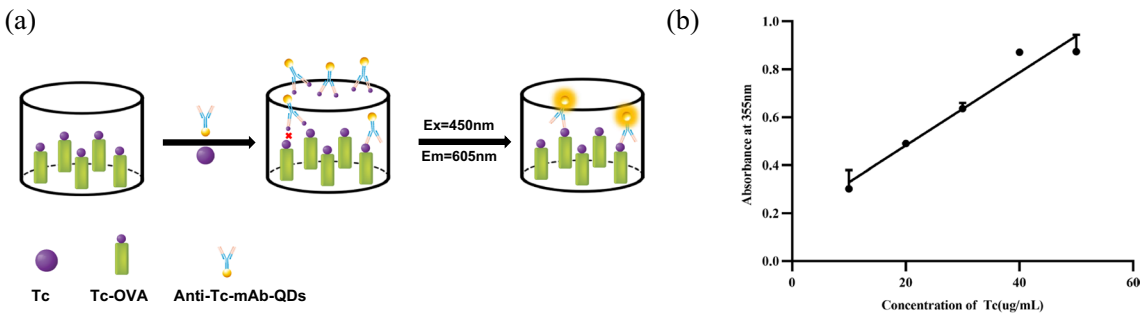

Fig. 6 Schematic representation and standard curve of the competitive fluorescence-linked immunosorbent assay (FLISA) for target analyte detection. (**a**) Schematic representation of FLISA competitive detection. (**b**) FLISA standard curve (y = -0.36X—0.24, $R.^2$ = 0.99)

Table 1 Comparison of the effectiveness between ic-ELISA and FLISA assays

Analytes	ic-ELISA (ng/mL)			FLISA (ng/mL)		
Tc	IC50	LOD	Range	IC50	LOD	Range
	9.88	2.02	3.00–32.50	8.77	0.69	1.30–59.22

the elimination of matrix effects. The results showed that for pork, chicken, and fish matrices, a tenfold dilution with PBS was effective, while for milk, egg, and honey matrices, dilutions of eightfold, 12-fold, and 13-fold with PBS, respectively, were required (Fig. 7). Under these conditions, the inhibition curves in the presence of matrix interference were in close agreement with the Tc standard curve, indicating the effective elimination of matrix effects.

Spiking recovery experiment and method validation

Based on the sensitivity of the FLISA method and the sample processing conditions, blank samples prepared earlier were spiked with Tc standard solutions at concentrations of 50, 100, 150, and 200 ng/mL. The spiked samples were tested in parallel using FLISA, and the recovery rates and RSD were calculated. The results showed that the recoveries of Tc in pork, chicken, fish, egg, milk and honey were in the range of 94.01%-110.19% with the relative standard deviations RSDs of 1.10%-11.39%, which were in good agreement with the results of HPLC–MS/MS (Table 3).

Table 2 Determination of the specificity of the FLISA assay

Competitor	IC50 (ng/mL)	CR (%)
Tetracycline	8.77	100
Chlortetracycline	11.475	76.43
Oxytetracycline	387.729	2.26
Minocycline	$>10^6$	<0.01
Tigecycline	$>10^6$	<0.01
Demeclocycline	$>10^6$	<0.01
Doxycycline	$>10^6$	<0.01

Precision and stability

The precision and stability of the FLISA method were evaluated. The results showed that the average IC50 value among batches was 8.93 ng/mL, the FV average was 44395, and RSD was less than 9.2%. The average IC50 value for different storage periods was 8.15 ng/mL, the FV average was 45236, and RSD was less than 6.8%. These results indicate that the established method exhibits good precision and stability (Table S2).

Blind sample testing

A total of six samples, including pork, chicken, fish, milk, eggs, and honey, were randomly purchased from local supermarkets and markets. After sample preparation, they were

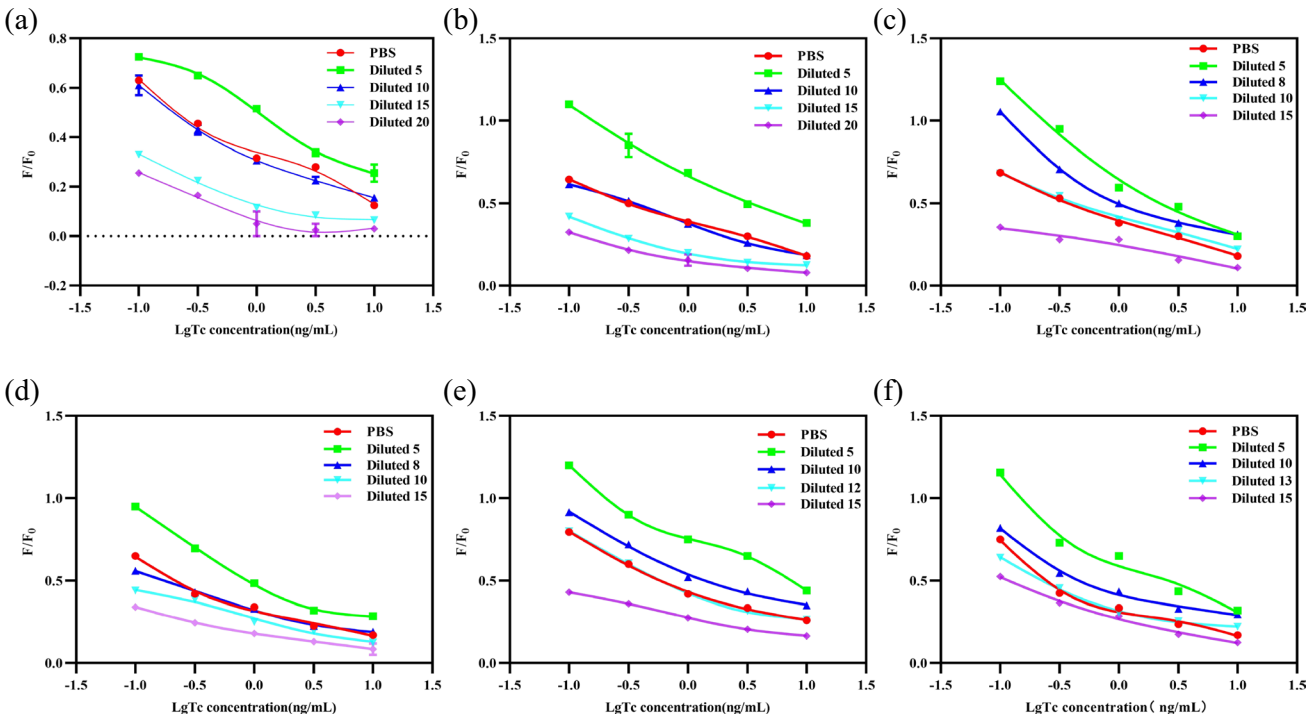

Fig. 7 Determination of optimal dilution of sample extracts. (**a**) The optimal dilution of the pork sample extract was tenfold. (**b**) Optimal dilution of chicken sample extract is tenfold. (**c**) The optimum dilution for fish sample extracts is tenfold. (**d**) The optimum dilution for milk sample extracts is eightfold. (**e**) The optimum dilution for egg sample extract is 12-fold. (**f**) The optimum dilution for honey sample extract is 13-fold

Table 3 Spiked recoveries and relative standard deviations for samples (n = 5)[a]

Samples	Spiked (ng/mL)	FLISA			HPLC–MS/MS		
		Found (M ± SD)[b]	Recovery (%)	RSD (%)	Found (M ± SD)[b]	Recovery(%)	RSD(%)
Pork	50	51.15 ± 4.74	102.31	9.28	54.16 ± 3.23	108.32	5.97
	100	99.27 ± 9.79	99.27	9.86	106.50 ± 3.16	106.50	2.97
	150	149.52 ± 9.86	99.68	6.59	153.67 ± 3.30	102.45	2.15
	200	206.16 ± 6.58	103.08	3.19	202.59 ± 5.90	101.30	2.91
Chicken	50	50.50 ± 5.75	101.00	11.39	52.20 ± 5.65	104.40	10.83
	100	98.96 ± 5.74	98.96	5.80	97.71 ± 6.04	97.71	6.18
	150	154.57 ± 7.35	103.05	4.76	151.53 ± 6.24	101.02	4.12
	200	211.75 ± 3.89	105.88	1.84	203.89 ± 6.88	101.95	3.38
Fish	50	54.63 ± 3.63	109.26	6.65	51.39 ± 6.40	102.77	12.45
	100	103.66 ± 9.37	103.66	9.04	99.26 ± 9.50	99.26	9.57
	150	145.10 ± 7.37	96.74	5.08	149.37 ± 6.74	99.58	4.51
	200	200.04 ± 9.84	100.02	4.92	203.93 ± 7.54	101.96	3.70
Milk	50	47.00 ± 1.75	94.01	3.73	52.50 ± 3.67	105.01	6.99
	100	112.65 ± 4.85	112.65	4.31	105.34 ± 4.97	105.34	4.72
	150	151.72 ± 7.61	101.15	5.02	148.26 ± 5.80	98.84	3.91
	200	213.19 ± 2.35	106.60	1.10	203.64 ± 6.47	101.82	3.18
Egg	50	51.88 ± 5.25	103.76	10.11	53.12 ± 4.00	106.24	7.53
	100	110.19 ± 4.71	110.19	4.27	98.84 ± 7.00	98.84	7.08
	150	158.12 ± 4.59	105.41	2.90	148.85 ± 7.52	99.23	5.05
	200	200.42 ± 10.24	100.21	5.11	205.99 ± 6.61	102.99	3.21
Honey	50	54.66 ± 3.89	109.32	7.11	45.67 ± 3.69	91.34	8.07
	100	102.26 ± 6.15	102.26	6.02	104.43 ± 6.40	104.43	6.13
	150	149.06 ± 11.21	99.37	7.52	149.37 ± 8.16	99.58	5.46
	200	214.29 ± 4.06	107.15	1.90	203.04 ± 5.17	101.52	2.55

[a]n = 5: All the above experimental results were obtained in five independent experiments

[b]*M* mean, *SD* standard deviation

subjected to testing. Among these, two samples showed detectable Tc residues, but both were below 500 ng/g, which is lower than the minimum limit of 2000 ng/g as stipulated by national standards. Therefore, all samples were considered negative. This result is consistent with the findings obtained using HPLC–MS/MS, indicating that the established direct FLISA method can be used for the determination of Tc residues in real samples.

Discussion

The increasing concerns surrounding antibiotic residues in animal-derived foods necessitate the development of efficient and reliable detection methods. This discussion explores the application of a Fluorescent Immunoassay (FIA) based on ZnCdSe/ZnS Quantum Dots (QDs) for the rapid and quantitative detection of tetracycline in such food products. This approach integrates nanotechnology and immunology, offering a promising solution to the challenges associated with antibiotic residue monitoring.

The emergence of quantum dots as fluorescent labels in immunoassays has revolutionized analytical techniques (Yang et al. 2019). ZnCdSe/ZnS QDs, with their exceptional optical properties, including high quantum yield and tunable emission spectra, provide a robust platform for sensitive and selective detection (Medintz et al. 2005). By harnessing the specificity of immunoreactions, this FIA method addresses the critical need for accurate and rapid quantification of tetracycline in animal-derived foods. Compared to traditional detection methods, such as HPLC and LC–MS/MS, the QDs-based FIA offers several advantages in terms of sensitivity, selectivity, and cost-effectiveness. The use of QDs as fluorescent labels enhances the sensitivity of the assay, enabling the detection of trace amounts of tetracycline in complex food matrices. Moreover, the specificity of the antibody-antigen interaction ensures high selectivity, minimizing the interference from other compounds present in the sample.

The successful implementation of the FIA relies on the synergistic combination of ZnCdSe/ZnS QDs and immunoassay principles (García-Fernández et al. 2014). The

immobilization of anti-tetracycline antibodies onto a solid support establishes a specific binding interface, facilitating the selective capture of tetracycline molecules. The subsequent detection, based on the fluorescence intensity of the QDs, enables precise quantification, allowing for a comprehensive analysis of tetracycline concentrations in complex food matrices (Resch-Genger et al. 2008). In our study, we optimized the experimental conditions, including the concentration of antibodies, incubation time, and temperature, to achieve the highest sensitivity and reproducibility. Under the optimized conditions, the developed FIA method exhibited a detection limit of 0.69 ng/mL and a quantitative detection range of 1.30 ~ 59.22 ng/mL, which is comparable or even superior to the performance of other reported methods (Xu et al. 2017; Gab-Allah et al. 2023; Wang et al. 2018).

The use of antibodies ensures the specificity of the assay, while the optical characteristics of ZnCdSe/ZnS QDs contribute to high sensitivity, allowing for the detection of trace amounts of tetracycline. The rapid response of quantum dots to changes in the binding environment ensures timely detection, making this FIA method suitable for high-throughput analysis in food safety laboratories (Zhang et al. 2011). The correlation between fluorescence intensity and tetracycline concentration provides a quantitative measure, enhancing the precision and reliability of the analytical results (Bustos et al. 2015). This method holds great promise for regulatory agencies, food producers, and consumers alike, offering a robust tool for the routine monitoring of tetracycline residues in animal-derived food products.

In addition to its superior performance, the QDs-based FIA method also represents a greener alternative to traditional detection methods. The use of ZnCdSe/ZnS QDs, which are less toxic compared to other types of QDs, such as CdSe and CdTe, reduces the environmental impact of the assay (Hardman 2006). Furthermore, the miniaturization of the assay format and the reduced sample volume required for analysis contribute to the overall sustainability of the method.

While the presented FIA method demonstrates significant potential, challenges such as matrix effects in complex food samples must be addressed. Future research endeavors could explore the extension of this methodology to detect other antibiotic residues and contaminants, thereby broadening its applicability in ensuring the overall safety of the food supply chain. Additionally, the development of portable and user-friendly devices incorporating this FIA method could facilitate on-site testing and real-time monitoring of antibiotic residues in food products.

In conclusion, the development of a Fluorescent Immunoassay based on ZnCdSe/ZnSQuantum Dots for the rapid quantitative detection of tetracycline in animal-derived foods represents a substantial advancement in analytical techniques for food safety monitoring. The integration of nanotechnology and immunology in this method showcases its potential as a reliable and efficient tool, contributing to the ongoing efforts to mitigate the risks associated with antibiotic residues in the food industry. The advantages of this method, including high sensitivity, selectivity, and cost-effectiveness, make it a promising alternative to traditional detection methods. Moreover, the use of green nanomaterials, such as ZnCdSe/ZnS QDs, aligns with the growing emphasis on sustainable and environmentally friendly analytical approaches.

The successful application of the developed FIA method to various animal-derived food samples, including pork, chicken, fish, milk, eggs, and honey, demonstrates its versatility and robustness. The high recoveries (94.01% to 110.19%) and low relative standard deviations (1.10% to 11.39%) obtained in the spiked sample analysis further validate the reliability and precision of the method. These results highlight the potential of the QDs-based FIA as a powerful tool for the routine monitoring of tetracycline residues in a wide range of food products.

The significance of this work extends beyond the specific detection of tetracycline in animal-derived foods. The principles and methodologies employed in this study can serve as a foundation for the development of similar immunoassays targeting other antibiotic residues and contaminants. By adapting the assay format and utilizing antibodies specific to other target analytes, this approach can be expanded to address a broader spectrum of food safety concerns. Furthermore, the successful demonstration of the QDs-based FIA in this context may inspire researchers to explore the application of nanomaterials in other areas of food analysis, such as the detection of pesticides, heavy metals, and mycotoxins.

Continued research and collaboration across interdisciplinary fields will be crucial for refining and implementing this innovative approach on a broader scale. Future efforts should focus on further optimizing the assay conditions, improving the stability and shelf-life of the reagents, and developing standardized protocols for sample preparation and analysis. Additionally, the integration of this FIA method with advanced instrumentation, such as microfluidic devices and automated sensing platforms, could enhance its throughput and ease of use, facilitating its adoption in various settings, from central laboratories to on-site testing facilities.

In summary, the development of a QDs-based FIA method for the rapid and quantitative detection of tetracycline in animal-derived foods represents a significant step forward in ensuring food safety and protecting public health. The advantages of this method over traditional approaches, coupled with its potential for broader application and adaptation, make it a valuable tool in the fight against antibiotic residues in the food supply chain. As research in this field continues to advance, it is anticipated that such innovative

analytical techniques will play an increasingly crucial role in safeguarding the quality and safety of our food products.

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Author contributions **CX** carried out the experimental work and wrote the manuscript. **JZ**, **YC**, **ZC**, **HX**, **YL**, **HL**, **CL**, **XZ** and **YZ** helped with the modification of the tetracycline group. **YQ** crunched the numbers and helped with the mouse immunization experiments. **GZ** and **AW** provided the initial idea, designed the study, and revised the manuscript. All authors contributed to the manuscript changes and read and approved the submitted version.

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Data availability The original contributions presented in the study are included in the article/supplementary material, and further inquiries can be directed to the corresponding authors.

Declarations

Ethics approval and Consent to Participate In this study, all animal experiments were approved by the Animal Ethics Committee of the College of Life Sciences, Zhengzhou University, and were conducted in compliance with the ARRIVE guidelines as well as the U.K. Animals (Scientific Procedures) Act of 1986 and associated guidelines, the EU Directive 2010/63/EU for animal experiments, and the National Research Council's Guide for the Care and Use of Laboratory Animals.

Conflict of interest The authors declare no competing interests.

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