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Highly sensitive visual detection of amantadine residues in poultry at the ppb level: A colorimetric immunoassay based on a Fenton reaction and gold nanoparticles aggregation

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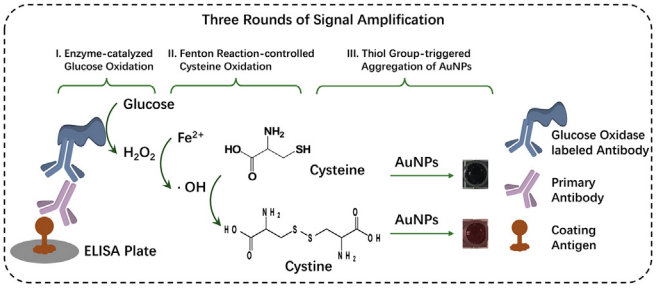
HIGHLIGHTS

- Combining ELISA with Fenton reaction and AuNPs aggregation.
- Assay sensitivity remarkably enhanced by a cascade reaction.
- Visual and quantitative detection of amantadine in poultry at the ppb level.

GRAPHICAL ABSTRACT



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ABSTRACT

Colorimetric biosensors for the on-site visual detection of veterinary drug residues are required for food control in developing countries and other resource-constrained areas, where sophisticated instruments may not be available. In this study, we developed a highly sensitive immunoassay for amantadine residues in poultry. By introducing a novel signal generation strategy into an indirect competitive immunoassay, a highly sensitive assay for amantadine residues in chicken was achieved for naked eye readout at the part per billion (ppb) level. Signal amplification was achieved in the designed immunoassay by combining conventional indirect competitive enzyme-linked immunosorbent assay, Fenton reaction-regulated oxidation of cysteine, and gold nanoparticle aggregation. Therefore, the cascade reaction remarkably enhanced the assay sensitivity and led to a pronounced color change from red to dark purple in the solution, which could be easily distinguished with the naked eye even at approximately 1 μg kg^{-1} in poultry muscle. Moreover, the color change can be quantitatively assayed with a classic high-throughput plate reader for contaminated poultry samples. The limit of detection (LOD) was 0.51 nM (0.095 ng mL^{-1}). The recovery rates for spiked chicken samples ranged from 78% to 84% with relative

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standard deviations <15%. Therefore, we propose that this immunoassay could be generally applicable for on-site detection in the field of food control.

1. Introduction

Amantadine (AMD) is one of the most important pharmaceutical agents for influenza infection in humans due to its interference with the M2 viral protein [1]. Because of its low price and availability, AMD has been and is still illegally used in the poultry industry [2]. Worryingly, its widespread use can increase the occurrence of bacterial resistant strains [2,3]. Countries including China and the United States have prohibited the use of AMD in poultry farms. Thus, a number of analytical methods have been established to determine AMD residues in chicken. Current approaches for determining AMD concentrations have primarily focused on instrument-based analytical techniques, such as gas chromatography (GC) [4], liquid chromatography (LC) [5], and high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) [6]. Although the detection, sensitivity, and precision of these assays are satisfactory, they require time-consuming sample pre-treatment, highly trained personnel, and expensive instruments, which interfere with the high-throughput, low-cost, and on-site detection that is necessary for food control in resource-constrained areas. Therefore, it is essential to develop straightforward, sensitive, and efficient analytical methods for AMD detection in foodstuffs.

Conventional immunoassays are highly sensitive and easy to operate and thus play a major role in the field of food analysis due to their ability to rapidly and effectively detect veterinary drug residues. Although aptamer- and molecularly imprinted polymer-based sensors represent alternatives to immunoassay formats and have been intensively studied [7,8], there are still some critical issues such as low affinity, poor assay stability, and involvement of chemical synthesis steps. When using conventional immunoassays for AMD detection, antibodies are a crucial factor for successful immune recognition. Until now, few research groups have prepared antibodies against AMD or developed common indirect competitive enzyme-linked immunosorbent assays (ELISAs) [9–11]. There are two colorimetric signal generation mechanisms for ELISA-based assays that are typically used as a readout: horseradish peroxidase (HRP)/tetramethylbenzidine (TMB) and alkaline phosphatase/p-nitrophenyl phosphate. Moreover, a microplate reader is an essential equipment for ELISA platforms, as the color changes of such substrates cannot be differentiated by the naked eye in the presence of low analyte concentrations, making it unsuitable for on-site detection. Emerging nanomaterials, such as gold nanoparticles (AuNPs), have shown great potential for developing colorimetric immunoassays, and could offer a visible and user-friendly signal readout [12]. Various strategies have been proposed to design AuNPs and other nanomaterials-based immunoassays that permit visual detection with high sensitivity by using enzyme-induced regulation of nanoparticles [13–17]. However, critical issues such as the requirement for complicated conjugation processes and time-consuming steps for nanoparticle modification have resulted in their unsuitability with current conventional immunoassay platforms [18–21]. Thus, the primary challenge to developing nanosensors to detect trace residues in foodstuffs by the naked eye is designing a straightforward signal generation strategy that can be directly applied to current immunoassay platforms.

Herein, we report a Fenton reaction and a glucose oxidase (GOx)-controlled signal generation mechanism that triggers AuNPs aggregation, resulting in a color change of the solution that is visible to the naked eye. Pioneering experiments demonstrated that cysteine (containing a thiol group) can assemble on the surface of AuNPs, leading to AuNPs aggregation through Au-S bonds and electrostatic interactions [22]. Whereas AuNPs aggregation cannot occur in the absence of free thiol groups [23]. Based on this principle, we developed a GOx-triggered cascade that was accelerated by a classic Fenton reaction system that could modulate AuNPs aggregation. Compared to the conventional immunoassay, the new immunosensor offered a much higher sensitivity by the naked eye, with a final concentration of 0.3 ng mL^{-1} which significantly improved the detection performance by 10-fold.

2. Materials and methods

2.1. Reagents and instruments

AMD was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Gold (III) chloride hydrate, Tween-20, and D-glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Amresco Inc. (Solon, OH, USA). Glucose oxidase-labeled goat anti-mouse IgG (gtAm-GOx) was purchased from Abcam (Cambridge, UK). AMD-OVA conjugates and monoclonal antibodies against AMD (mAb 3F2) were produced in our laboratory as previously described [24]. TMB solutions were purchased from Beyotime Biotechnology (Shanghai, China). All other analytical grade reagents required for the experiments were obtained from Sigma-Aldrich.

2.2. Synthesis of AuNPs

AuNPs were prepared by the citrate reduction method using previously published methods [25]. Briefly, 100 mL of 1 mM $HAuCl_4$ aqueous solution was heated under reflux. After boiling, 10 mL sodium citrate (38.8 mM) was added to the rapidly stirring $HAuCl_4$ solution. The reaction mixture was boiled for 15 min and allowed to cool to room temperature. Then, the AuNPs were collected by filtration through a 0.22-μm polyethersulfone membrane. To yield well-dispersed AuNPs, 2 mL of 1% Tween-20 was transferred into 50 mL of AuNPs [26]. The final AuNPs solution was stored at 4 °C until use.

2.3. Fenton reaction-modulated AuNPs aggregation

First, we tested a series of cysteine concentrations to optimize the conditions. The system contained 100 μL AuNPs, 20 μL $FeSO_4$ (1 mM in H_2O), and 30 μL distilled water. Then, different concentrations of cysteine were added to reach a final volume of 200 μL. After a 40 min incubation at room temperature, the AuNPs aggregation was saturated, which is comparable to the results of cysteine-stimulated aggregation of AuNPs [27]. The UV–vis spectrum of the solution was measured on a SpectraMax M5 microplate reader (Molecular Devices San Jose, CA, USA).

Second, the Fenton reaction-induced aggregation of AuNPs was evaluated. H_2O_2 was diluted with deionized water to reach final