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Research Article

Ultrasensitive Nano-ELISA for Detecting Sulfadimethoxine in Chicken Tissue

Chi-Fang Peng,[1] Xiao-Hui Duan,[1] Qiu-Li Pan,[1] Li-Qiang Liu,[1] and Feng Xue[2]

[1] *State Key Lab of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, China*
[2] *Animal, Plant & Food Inspection Center, Jiangsu Entry-Exit Inspection and Quarantine Bureau, Nanjing 200001, China*

Correspondence should be addressed to Chi-Fang Peng; pcf@jiangnan.edu.cn

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It is challenging to obtain a highly sensitive enzyme-linked sorbent immunoassay (ELISA) method for the rapid screening of veterinary drug residue in animal tissues. Here we present that a simple and sensitive detection for sulfadimethoxine (SDM) residue in animal tissues was achieved by utilizing a bioconjugate of gold nanoparticles and enzyme-labeled antibody as signal probe in enzyme-linked sorbent immunoassay (ELISA). The developed nano-ELISA has increased the sensitivity of a traditional ELISA for SDM by 20-fold. The sensitivity of this ELISA was 5 pg/mL in buffer, and the detection limit (LOD) of 0.2 μg/kg can be obtained after chicken liver was simply extracted by buffer. This simple and sensitive method can be used to improve the sensitivity of ELISA methods for various small molecule contaminants.

1. Introduction

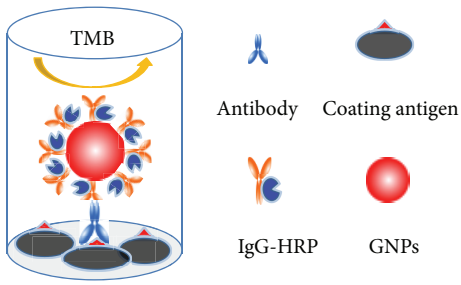
Sulfonamides are widely used to treat bacterial and protozoan infections in food animals. Their pharmacological activity is due to their ability to mimic p-aminobenzoic acid (PABA) and to inhibit the early stages of folic acid synthesis in bacteria and in various protozoans [1]. However, sulfonamides can be accumulated in the body of people who consumed products from those excessively drug-treated animals, resulting in the development of antibiotic-resistant pathogenic bacteria. To minimize this risk, a maximum residue limit of 0.1 mg kg^{-1} has been established for total or individual sulfonamides in food samples, including meat and milk, by Codex Alimentarius Commission in European and American countries [2]. Sulfadimethoxine (SDM) is a typical example of sulfonamides which are widely used antibiotics worldwide. Many cases about administrating SDM excessively have been reported for the prevention and treatment of infections in chicken, swine, and cattle [2–5].

Conventional residue methods for the detection of sulfonamides in animal tissues include bioassays, thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [1, 5–8]. In an effort to increase both method sensitivity and sample throughput, enzyme-linked immunosorbent assays (ELISAs) have been developed for several of the sulfonamides [1, 9–11]. Although high sensitivity has been achieved in the above-mentioned immunoassays, accomplishing the detection of veterinary drug residue in animal tissues simply and sensitively by ELISA method is still challenging due to serious matrix interference [12, 13]. Nevertheless, developing highly sensitive ELISA method can avoid this problem by diluting the sample extract and decreasing the matrix interference obviously [12, 14–16].

Gold nanoparticles (GNPs) with outstanding characteristics have attracted great interest in biosensors recent years [17–20]. In this work, a novel nano-ELISA was used for rapid and sensitive detection of SDM, as a model compound for veterinary antibiotics. Scheme 1 that shows an immune-reaction nanocomplex forms in the surface of microplate wells. Combing a traditional ELISA format and a GNP-antibody-enzyme bioconjugate as label, this nano-ELISA can achieve very low detection limit of 0.2 ng/g SDM in chicken tissue by implementing as simply as the traditional ELISA.

2. Experimental

2.1. Chemicals and Reagents. Monoclonal mouse antibody against SDM was produced by immunizing balb/c mice with



Scheme 1: The immune-reaction nanocomplex forms in the surface of microplate wells.

SDM-BSA and hybridoma cell cloning technique in our laboratory [21]. SDM was purchased from Aladin Regent (Shanghai). Ovalbumin (OVA), 3,3′,5,5′-tetramethylbenzidine (TMB), chloroauric acid (HAuCl4), and trisodium citrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The peroxidase-conjugated goat anti-mouse IgG (IgG-HRP) was purchased from Kangchen Biotechnology Company (Shanghai, China). Control chicken liver tissue was obtained from the Testing Center of Animals, Plants, and Food, Jiangsu Entry and Exit Inspection, and Quarantine Bureau. Microtiter plates were obtained from Costar Group, Inc. (Bethesda, MD, USA). Other chemical regents all were of analytical grade and from Shanghai Chemical Reagents Company (Shanghai, China). All buffers used in the experiments were prepared using Milli-Q water by Milli-Q system (Millipore, Bedford, MA).

2.2. Solutions and Buffers. Coating buffer, 50 mmol/L carbonate buffer (pH 9.5); dilution buffer (phosphate buffered saline (PBS)), 10 mmol/L sodium phosphate buffer (pH 7.4) containing 140 mmol/L NaCl; washing buffer, PBS containing 0.05% (v/v) Tween 20 (PBST); TMB solution, 50 mmol/L sodium citrate buffer (pH 5.0) containing 0.01% (w/v) TMB, and 0.005% (v/v) H_2O_2 were used.

2.3. Preparation of Conjugate of Hapten and Protein. The hapten of SDM and protein conjugate were prepared according to [1]. Briefly, 10 mg SDM was dissolved in warm 0.1 N sulfuric acid (4 mL, 70°C). The solution was then cooled in an ice bath, and 1 mL of freshly prepared sodium nitrate (19 mg/mL) was added dropwise over 3 min and incubated for an additional 3 min. A solution of OVA (100 mg in 4 mL of 0.5 M borate, pH 9.4) was cooled to 0°C, and the diazotized hapten solution was added to the cooled OVA solution over a period of 15 min. The pH of the solution was maintained between 9.0 and 9.5 using sodium hydroxide (1 M). After 4 h, the reaction mixture was stored at room temperature, exhaustively dialyzed against 10 mM phosphate buffer, pH 7.2, (9 × 2 L) at 4°C, and stored at −20°C prior to use.

2.4. Synthesis and Characterization of Gold Nanoparticles. Gold nanoparticles (GNPs) (15 nm) were synthesized by reducing tetrachloroauric acid with trisodium citrate [22]. All glassware and magnetic stir bars used in the synthesis were thoroughly cleaned in aqua regia. 100 mL of 0.1% $HAuCl_4$ solution was boiled with vigorous stirring in a 250 mL round-bottom flask. Two mL of 1% trisodium citrate solution was then added quickly to the boiling solution resulting in a color change from pale yellow to dark red and indicating the formation of GNPs. The solution was maintained for 10 min at boiling temperature and then stirred to cool down. The synthesized GNPs were characterized using UV-vis spectroscopy (Figure 2) and dynamic laser scattering (DLS) (Figure 3) to analyze their size.

2.5. Preparation of the GNP-IgG-HRP Conjugate. The GNPs-IgG-HRP conjugate was prepared as follow. Precisely, 1 mL of GNP (15 nm) solution was mixed with 1, 2, or 3 μL of K_2CO_3 (200 mM), and 100 μL of IgG-HRP solution at a concentration of 100 μg/mL was added to 1 mL of the GNP solution. The mixture was stirred for 30 min, and then it was centrifuged at 13 000 rpm for 15 min at 4°C to remove the excess of antibody. The clear supernatant was carefully removed, and the precipitated gold conjugates were resuspended in 1 mL of PBS buffer (0.01 M, pH 7.4) and stored at 4°C. The stabilities of these conjugates were evaluated by adding 100 uL of NaCl (10%) and keeping at 4°C for 24 hours to observe their color change. It was found that the conjugates prepared with adding 1-2 μL of K_2CO_3 appeared blue to some extent, while the conjugate prepared with adding 3 μL of K_2CO_3 still appeared red. Finally, this conjugate, prepared with adding 3 μL of K_2CO_3, was used for further application. The conjugate was also characterized using UV-vis spectroscopy (Figure 2) and DLS (Figure 3) to analyze the size.

2.6. Nano-ELISA. The protocol of the nano-ELISA was similar to traditional indirect ELISA method except that the IgG-HRP was replaced with the GNPs-IgG-HRP conjugate. The general procedure adopted to develop the assay was as follows. 100 μL of SDM-OVA (10 μg/mL) was added to each well of 96-well microplate and immobilized overnight. Excess coating antigen was removed by decanting the plate content and washing the plate wells. The remaining sites in the well were blocked by incubating 200 μL of blocking buffer (1% BSA in PBST) for 2 h at room temperature. After decanting blocking buffer and washing, 50 μL of SDM standard solution and 50 μL of anti-SDM antibody solution diluted by antibody dilution buffer (PBST containing 0.1% gelatin) were added to the wells and then incubated at 37°C for 30 min. After the solution in wells was decanted and the wells were washed, 100 μL of diluted GNPs-IgG-HRP conjugate was added to each well. After incubation for 30 min, the wells were washed. HRP substrate, TMB and H_2O_2 solutions, were added and color was developed for 15 min. Then the reaction was stopped by adding 2 M sulfuric acid (100 μL) to each well. The optical density was read at 450 nm with a microplate reader within 5 min after stopping the reaction.

2.7. Sample Pretreatment and Validation of the Nano-ELISA. The sample pretreatment method was slightly modified according to [7]. Control chicken liver tissues, confirmed without SDM contamination by LC-MS/MS, were cut up

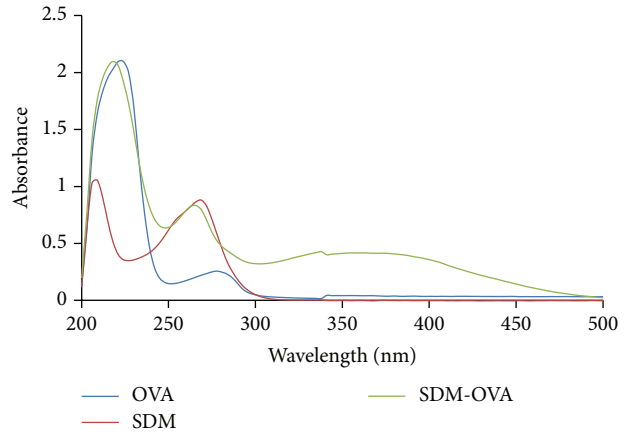


Figure 1: UV-vis spectrum of SDM-OVA conjugate.

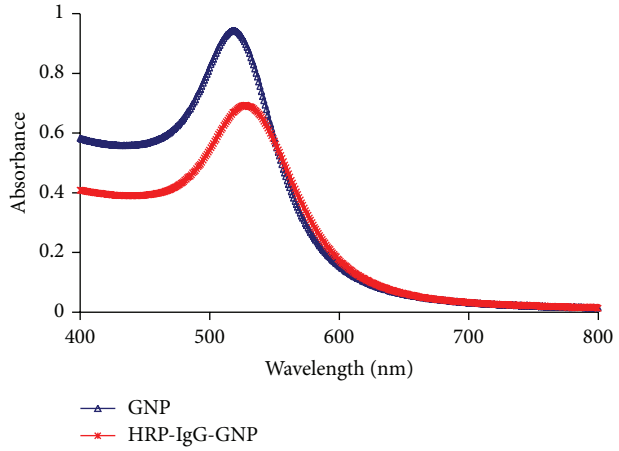


Figure 2: UV-vis spectrum of GNP and HRP-IgG-GNP.

and hoogenized. To each 2 g sample, certain SDM standard solution (0.1 mg/mL) was added to obtain SDM spiked samples. After standing for 30 min, the spiked samples were homogenized with 5 mL PBS (pH 9.0, 0.01 M) at 10000 rpm for 2 min and then centrifuged at 5000 rpm for 10 min. The supernatants were diluted 1 : 20 with PBS (0.01 M, pH 8.0), and 50 μL aliquots were added to the microplate wells. Then anti-SDM antibody was added for the immunoreaction. Other steps were the same with the protocol of the above nano-ELISA. Meanwhile, the stocked SDM standard solution were added directly to the buffer extract from the control chicken liver tissue and prepared SDM standards (0.05–20 ng/mL) containing the sample matrix. These SDM standard solutions also will be utilized for calibration of the nano-ELISA.

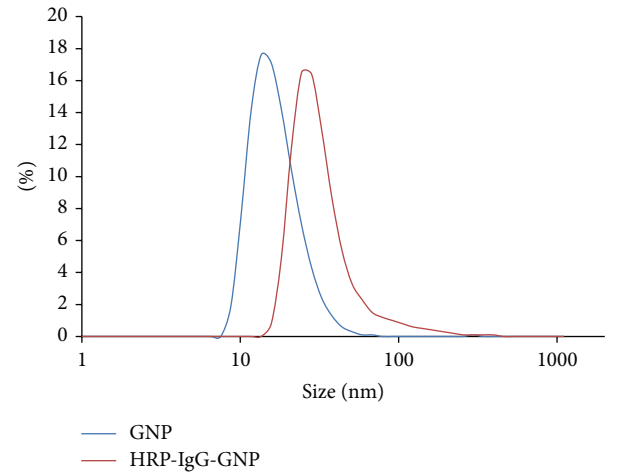


Figure 3: Size distributions of GNP and HRP-IgG-GNP.

3. Results and Discussion

3.1. Characterization of SDM-OVA Conjugate. The conjugate of SDM and OVA, as coating antigen, was qualitatively analyzed by comparing the UV-vis spectrum of the SDM-OVA with those of OVA and SDM. The SDM-OVA conjugate had two absorption peaks (Figure 1), one of which was 270 nm (λmax) and also the characteristic absorption peak of SDM. Between 300 nm and 500 nm, a broad absorption peak was the characteristic absorption peak of diazo. The results demonstrated SDM and OVA were conjugated successfully.

3.2. Characterization of GNPs and GNPs-IgG-HRP Conjugate. The initial maximum absorbance peak of the GNPs at 518 nm was shifted to 530 nm after conjugated with IgG-HRP molecules (Figure 2). This was the phenomenon that the plasma absorbance peak of GNPs increases with the size of GNPs increasing. DLS analysis showed that the hydrate sizes of the GNPs and GNPs-IgG-HRP conjugate were 18 nm and 36 nm, respectively (Figure 3). After combining IgG-HRP, the conjugate size increased by 18 nm, which was close to the twofold of IgG-HRP size. This size expansion demonstrated that the combination of GNPs and IgG-HRP was compact and stable. It was important to note that the GNPs-IgG-HRP conjugate was also monodisperse (data not shown), which will contribute to the reproducibility of the GNPs-based ELISA method.

3.3. The Performance of the Nano-ELISA. GNPs with high surface areas offer a possibility to design probes for signal amplification by immobilizing multiple kinds of biomolecules to the surface of GNPs [19, 23]. For application of the GNPs-IgG-HRP conjugate in ELISA method, the concentrations of coating antigen, anti-SDM antibody, and the GNPs-IgG-HRP conjugate have to be screened. Blocking buffer containing proteins or polymers (ovalbumin, gelatin from pork skin, polyvinyl pyrrolidone (PVP, average mw 40 kDa), PEG-10000) was compared. Blocking buffer containing gelatin (0.1%) output highest signal to noise (S/N) (data not shown). Under optimized concentrations of coating antigen (6.0 μg/mL), anti-SDM antibody (2.0 μg/mL) and the GNPs-IgG-HRP conjugate (30 fold dilution), the sensitivity (when A/A_0% was 90%) could reach 5 pg/mL, which was 20 times more sensitive than that of the traditional ELISA for SDM (Figure 4). The linear range of nano-ELISA was

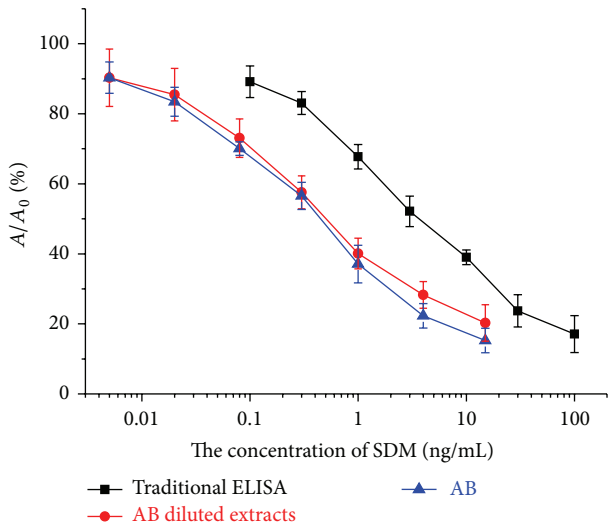


Figure 4: Calibration curves for the traditional and nano-ELISA methods for SDM obtained with assay buffer (AB) or with AB dilution of control liver extracts ($n = 4$).

0.005~5 ng/mL ($R^2 > 99\%$). These results demonstrated that multiple IgG-HRP molecules loading onto the surface of GNPs had rendered the conjugate producing signal amplification function. The signal amplification effect in this method was close to that of other GNPs-based ELISAs for lead ion [19].

This amplification was reasonable, because 20 IgG-HRP molecules, approximated to a sphere of a radius of 5.6 nm, can be bound to the surface of a gold nanoparticle (15 nm), which was estimated by using the geometrical model of sphere packing around a single central sphere of radius 7.5 nm (gold nanoparticle) [24]. If IgG-HRP is elongated, more than 20 IgG-HRP molecules will be probably packed on the surface of a gold nanoparticle (15 nm) [25]. It should be noted that the performance of this GNPs-based ELISA can be improved further through more elaborate design, for example, optimizing the size of GNPs, adopting other kind of bigger nanoparticle, or directly conjugating IgG and HRP to improve the loading of HRP molecules per GNP.

3.4. Validation of the Nano-ELISA. To investigate the performance of this nano-ELISA method for real samples, pretreated control chicken liver tissues were utilized to prepare SDM standard solutions and first tested by this method. It showed that the matrix effect can be eliminated almost completely after 20-fold dilution and the standard curve obtained using this diluted matrix control was almost identical to the curve obtained using assay buffer (AB) alone (Figure 4).

The spiked chicken liver tissues were tested by the established sample pretreatment protocol. The limits of detection (LOD) for SDM in chicken liver were calculated by taking the mean value of 10 blank samples plus 3 times standard deviations of the mean value and found to be 0.2 μg/kg. The chicken liver samples were spiked with 1, 6, and 40 μg/kg SDM, respectively, and extracted as mentioned before. The detection recoveries were between 83.7% and 95.2% (Table 1) when SDM spiked concentrations were from 1 μg/kg to 40 μg/kg and the extracts were diluted 1 : 20, which demonstrated that this nano-ELISA was reliable for SDM residue detecting in chicken liver.

Table 1: Recovery of SDM in chicken liver detected by nano-ELISA ($n = 4$).

Spiked concentration (μg/kg)	Results measured (μg/kg)	Recovery (%)	Relative derivation (%)
1.0	0.837 ± 0.076	83.7	9.3
6.0	5.71 ± 0.34	95.2	6.1
40	36.15 ± 3.25	90.3	9.0

4. Conclusions

In this work, stable conjugate was obtained by facile preparation of GNPs-IgG-HRP conjugate. Appling this conjugate as probe in traditional ELISA, we developed a nano-ELISA method and achieved a highly sensitive detection of SDM in chicken liver tissues. Comparing to traditional ELISA, this nano-ELISA remains simple protocol but is 20 times more sensitive than the later. This novel nano-ELISA method will be useful in screening detection of SDM in animal food and also can be extended to develop detecting methods for other low-molecular weight molecules.

Acknowledgments

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