Analytica Chimica Acta 812 (2014) 236–242

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

A competitive immunoassay for sensitive detection of small molecules chloramphenicol based on luminol functionalized silver nanoprobe

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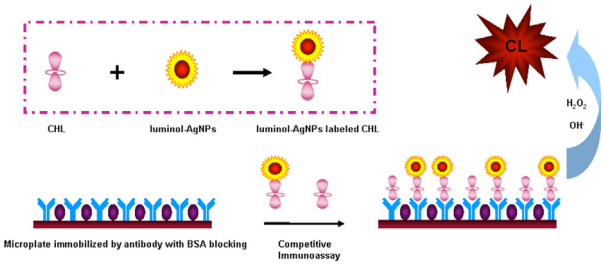
HIGHLIGHTS

- Novel silver nanoparticles functionalized by luminol are used for the labeling.
- The labeling procedure is simple, convenient and fast.
- A competitive CL immunoassay has been developed for the detection of CHL.
- The immunoassay is simple, fast, sensitive and selective.
- It is of application potential for the determination of CHL in foodstuffs.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 October 2013
Received in revised form 6 January 2014
Accepted 8 January 2014
Available online 15 January 2014

Keywords:
Chemiluminescence
Luminol functionalized silver nanoprobe
Chloramphenicol
Immunoassay

ABSTRACT

Chloramphenicol (CHL) as a broad-spectrum antibiotic has a broad action spectrum against Gram-positive and Gram-negative bacteria, as well as anaerobes. The use of CHL is strictly restricted in poultry because of its toxic effect. However, CHL is still illegally used in animal farming because of its accessibility and low cost. Therefore, sensitive methods are highly desired for the determination of CHL in foodstuffs. The immunoassays based on labeling as an important tool have been reported for the detection of CHL residues in food-producing animals. However, most of the labeling procedures require multi-step reactions and purifications and thus they are complicated and time-consuming. Recently, in our previous work, luminol functionalized silver nanoparticles have been successfully synthesized, which exhibits higher CL efficiency than luminol functionalized gold nanoparticles. In this work, the new luminol functionalized silver nanoparticles have been used for the labeling of small molecules CHL for the first time and a competitive chemiluminescent immunoassay has been developed for the detection of CHL. Owing to the amplification of silver nanoparticles, high sensitivity for CHL could be achieved with a low detection limit of 7.6×10^{-9} g mL^{-1} and a wide linear dynamic range of 1.0×10^{-8}–1.0×10^{-6} g mL^{-1}. This method has also been successfully applied to determine CHL in milk and honey samples with a good recoveries (92% and 102%, 99% and 107% respectively), indicating that the method is feasible for the determination of CHL in real milk and honey samples. The labeling procedure is simple, convenient and fast, superior to previously reported labeling procedures. The immunoassay is also simple, fast, sensitive and selective. It is of application potential for the determination of CHL in foodstuffs.

1. Introduction

Chloramphenicol (CHL) as a very effective broad spectrum antibiotic is widely used in veterinary practice as feed additives for growth promotion because of its low cost. However, research has shown that it can lead to serious adverse reactions and side effects in humans such as aplastic anemia, which is often fatal [1]. These

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http://dx.doi.org/10.1016/j.aca.2014.01.021

potential hazards have led to a prohibition of its use in the United States and European Union in 1994 in order to protect humans from the potentially harmful antibiotic residues [2]. However, CHL is still illegally used in animal farming because of its accessibility and low cost and its residues have been found in various food samples, such as muscle, shrimp, milk and honey [3]. In order to effectively monitor the occurrence of residues of CHL, specific and sensitive analytical methods are required.

In recent years, the immunoassays based on labeling as an important tool have been reported for the detection of CHL residues in food-producing animals. The labeling of small molecules is very difficult work. A great effort has been made for labeling of CHL. Park et al. [4] reported a direct competitive chemiluminescent (CL) immunosensor by using horseradish peroxidase (HRP) as a label. The labeling procedure was accomplished according to the activated ester method reported previously [5]. The CHL succinate solution first mixed with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate and *N*-hydroxysuccinimide solution, and then HRP was added into the activated CHL succinate to obtain the CHL-HRP. Zhang et al. [6] developed a competitive immunoassay by fluorescently labeled CHL hapten, using capillary electrophoresis (CE) with a laser-induced fluorescence (LIF) detector. The CHL hapten was coupled to keyhole limpet hemocyanin (KLH) as the immunogen and was then mixed with fluorescein thiocarbamyl ethylenediamine (EDF) in dimethylformamide to form an EDF labeled CHL. Gasilova et al. [7] presented a fluorescence polarization immunoassay of the CHL using fluoresceinisothiocyanate (FITC) as a label. The labeling procedure was completed by direct grafting of FITC label to an aminochloramphenicol in methanol and triethylamine blend solution. Jarujamrus et al. [8] described the feasible method for analysis of CHL using AuNPs as labels by coupling competitive immunoassay with inductively coupled plasma mass spectrometry (ICP-MS). The labeling procedure was carried out by the adsorption of CHL-BSA (bovine serum albumin) protein to AuNPs. Yuan et al. [9] reported a direct competitive gel-based visual immunoassay by using HRP as a label. The labeling procedure was similar with Zhang et al. [6] by using HRP instead of EDF to form a HRP labeled CHL. However, most of labeling procedures requires multi-step reactions and purifications and thus they are complicated and time-consuming. Therefore, it is necessary to exploit a sensitive and simple labeling strategy for the CHL detection.

In recent years, a variety of CL or electrogenerated chemiluminescence (ECL) functionalized nanoparticles were synthesized [10–14] and have been successfully used as labels for the detection of macromolecules like DNA and protein [15–22]. However, the functionalized nanoparticles were rarely reported for labeling of small molecules. Recently, novel silver nanoparticles functionalized by luminol have been synthesized [12] by our group. In this work, they are successfully used for the labeling of small molecule CHL. Based on this luminol functionalized silver nanoprobe, a competitive immunoassay for sensitive detection of small molecules CHL was developed. The conditions for the detection of target CHL were optimized and the analytical performance of the resulting CHL immunoassay was studied. Finally, the applicability of the strategy in real milk and honey samples was explored.

2. Experimental

2.1. Chemicals and solutions

$AgNO_3$ and absolute ethanol (G, R) were purchased from Shanghai Reagent (Shanghai, China). Anti-chloramphenicol monoclonal antibody and CHL were purchased from Huaan Magnech Bio-Tech Co., Ltd. An $AgNO_3$ solution of 5×10^{-3} mol L^{-1} was prepared by dissolving $AgNO_3$ in purified water and stored at 4 °C. A stock solution of luminol (1.0×10^{-2} mol L^{-1}) was prepared by dissolving luminol (Sigma) in 0.1 mol L^{-1} NaOH aqueous solution. Working solutions of H_2O_2 were prepared fresh daily from 30% (v/v) H_2O_2 (Xin Ke Electrochemical Reagent Factory, Bengbu, China). Bovine serum albumin (BSA) was purchased from Solarbio (Beijing, China). The buffer solutions used in the experiment included 0.05 mol L^{-1} carbonate buffer (CBS, $NaHCO_3$–Na_2CO_3), pH 7.4 phosphate saline buffer (PBS). All glassware used in the following procedures was cleaned in a bath of freshly prepared HNO_3/HCl (3:1, v/v), rinsed thoroughly in redistilled water, and dried prior to use. All other reagents were of analytical grade. Ultra-pure water was prepared by a Millipore Milli-Q system and used throughout. The food used for the analysis (milk and honey) was purchased from local supermarket.

2.2. Preparation of luminol-AgNPs labeled CHL

Luminol-AgNPs were prepared by one-pot method as follows [12]: first, a 2 mL portion of $AgNO_3$ solution with the concentration of 5.0×10^{-3} mol L^{-1} was mixed with 5 mL ultrapure water and 9 mL absolute ethanol. While stirring vigorously at 25 °C, 0.5 mL portion of luminol stock solution (1.0×10^{-2} mol L^{-1}) was added rapidly; the solution was stirred for 2 h, during which time a color change from colorless to primrose yellow to deep yellow, indicating the formation of silver nanoparticles. The unreacted reagents were removed via dialysis for 2 days with ultrapure water about six times under stirring by use of a 3500 molecular weight cut-off dialysis membrane to obtain luminol-AgNPs. The morphologies of luminol-AgNPs were characterized by transmission electron microscope (TEM). As shown in Fig. 1B, spherical nanoparticles with a size of approximate 25 nm were obtained.

The luminol-AgNPs with good CL property were prepared as described previously and stored at 4 °C. Luminol-AgNPs labeled CHL was prepared as follows: 2 mL of luminol-AgNPs colloid and 1 mL of CHL solution (1.0×10^{-6} g mL^{-1}) were mixed and stirred for 40 min at room temperature for effective loading of the CHL onto the luminol functionalized silver nanoparticles. After that, a final concentration 5% of BSA was added into the mixed solution to block the excessive luminol-AgNPs, then incubated for 30 min at 37 °C and stored at 4 °C before use. A schematic for the preparation of the luminol-AgNPs labeled CHL is given in Fig. 1A. The morphologies of luminol-AgNPs labeled CHL were characterized by TEM as shown in Fig. 1C.

2.3. Immobilized antibody onto the microplate

Coating involves the interaction between the solid phase surfaces and reagents in immunoassay. First, according to the introduction supplied by the company, each well of the microplates was washed with 100 μL of 1% (w/v) BSA for 5 min for three times and the plate activated with BSA reacted more stably and actively. Then, each well of the microplates was coated with 100 μL of the CHL antibody (2.0×10^{-6} g mL^{-1}) in CBS buffer solution. The plate was allowed to stand sealed at 4 °C overnight. After that, the plate was washed by PBS buffer solution three times and then 100 μL of the blocking BSA (5%) was added into each well and the plate was put at 37 °C for 30 min to block the active sites on the plate. Subsequently, the plate was washed by PBS buffer solution in triplicate and gently tapped against tissue paper to remove all fluid and stored at 4 °C for further use.

3. Competitive immunoassay

During the detection of target CHL, a mixture of 100 μL luminol-AgNPs labeled CHL solutions with 100 μL of CHL solution, after