

## Highly efficient and sensitive detection of chloramphenicol based on chemiluminescence immunoassays with functionalized $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{Au}$ magnetic nanoparticles

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**Abstract.** To avoid the long time of dialysis process, a novel Strategy that Bovine serum albumin (BSA) was immobilized on Au-MNPs at first and afterward the CAP molecules were immobilized on the BSA in sequence has been applied to Chloramphenicol (CAP) detection with chemiluminescent immunoassays (CLIA) based on functionalized  $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{Au}$  magnetic nanoparticles. More importantly, this designed method was successfully applied to CAP detection with the highly efficient and sensitive. With the functionalized gold magnetic nanoparticles (Au-MNPs). Samples were detected utilizing the two different chemiluminescence immunoassays, with limits of detection (LODs) of  $0.07 \text{ pg mL}^{-1}$  for Method I and  $0.06 \text{ pg mL}^{-1}$  for Method II and a 50% inhibition concentration ( $\text{IC}_{50}$ ) value of  $0.086 \text{ ng mL}^{-1}$  for Method I and  $0.14 \text{ ng mL}^{-1}$  for Method II for CAP respectively. Therefore, such chemiluminescence immunoassays method could be easily adapted for small molecule detection in a variety of foods using Au-MNPs to conventional ILESA immunoassays in CAP detection.

### 1. Introduction

Chloramphenicol (CAP) was the first mass-produced antibiotic, as an effective drug against typhoid, which was extensively used as an antibiotic against bacterial infections in animals. Nevertheless, The use of chloramphenicol is prohibited when breeding animals, because chloramphenicol (ingested via foodstuffs) is highly toxic to humans, such as bone marrow depression and aplastic anemia[1]. Therefore, a minimum required performance limit (MRPL) of  $0.3 \text{ } \mu\text{g kg}^{-1}$  for CAP was set by the European Commission in products of animal origin (Commission decision, 2003/181/EC)[2]. Nowadays, In many countries, this level has been set at zero. The most commonly used analytical methods have been described for the detection of FQs in tissues, including high-performance liquid chromatography[3], liquid chromatography-mass spectrometry (LC-MS)[4], and LC-MS/MS[5]. However, these methods require expensive instruments and time-consuming sample preparation steps, thus not suitable for detection of large number of samples[6]. therefore, there is an imperative for rapid, sensitive, and economical methods for detecting CAP and the other antibiotic residues in food.

Chemiluminescence (CL) reaction can applied to lots of analytical biochemistry with their superior, such as the sensitivity, short-time running and the wide linear range[7]. Nowadays, CL immunoassays (CLIA) consisting of CL systems and immunoreactions, was becoming widely popular applied to food industry for detection of the antibiotics and pesticides[8]. As a new luminescence reagent, 2',6'-DiMethylcarbonylphenyl-10-sulfo propyl acridinium-9-carboxylate-4'-NHS Ester (NSP-DMAE-NHS) demonstrates the higher photo quantum efficiency. It can emit light in two seconds and full automation. Therefore, it has been exploited in CLIAs to gain higher sensitivity for rapid screening in food safety detection[9].

Magnetic nanoparticles (MNPs) have been applied to immunoassays to increase sensitivity,

specificity and without effect the antigens or antibodies specificity. Besides, the coupled magnetic particles could be easily separated and enriched in a magnetic field[10].The composite particles, Gold-coated magnetic nanoparticles (Au-MNPs), have become populer with the outstanding characteristics that they connected the biomolecules (such as DNA, antibody) on gold surface without effect their biological activities. Therefore, many methods of CAP detection have utilized MNPs and Au-MNPs in order to enhance sensitivity and reduce detection time.

In this study, Au-MNPs based CLIA using NSP-DMAE-NHS as luminescence reagent for CAP rapid detection has been developed. BSA was immobilized on Au-MNPs at first and CAP base or CAP succinate were conjugated with immobilized BSA in sequence. The anti-CAP antibodies were labeled by NSP-DMAE-NHS. Importantly, Under optimized assay conditions, a wide linear detection range (from  $10^{-5}$  to  $10^{-3}$  ng mL<sup>-1</sup>) was achieved with a detection limit down of 0.07 pg mL<sup>-1</sup> for Method I and 0.06 pg mL<sup>-1</sup> for Method II. Further more, this methods can be useful for the other antibiotics.

## 2. Materials and methods

### 2.1 Reagents

CAP mAb was purchased from Bioss Co., Ltd ( Beijing, China ). (2', 6'-DIMethylcarbonylpheny 1-10-sulfopropylacridInum-9-carboxylate-4'-NHSEster ( NSP-DMAE-NHS ), chloramphenicol (CAP) were purchased from Mater Win new materials Co., Ltd ( Shanghai, China). 3-aminopropyltrimethoxysilane (APTES) and bovine serum albumin (BSA) were purchased from J&K Scientific Co., Ltd (Beijing China). L-lysine and Sephadex G-50 were purchased from Seebio (Shanghai, China). FeCl<sub>3</sub>·6H<sub>2</sub>O, HAuCl<sub>4</sub>·3H<sub>2</sub>O, tetraethoxysilane (TEOS) and sodium acetate were purchased from Tianjin Tianli Chemical Regents Ltd. 1-ethyl-3-(3-dimethylamino-propyl )-carbodiimidehydrochloride (EDC), N-hydroxysuccinimide (NHS), N,N-dimethylformamide (DMF) and 2-(N-Morpholino) ethanesulfonic acid (MES) were purchased from Sigma Company (China).

### 2.2 Construction of luminescent measurement system

The diagram of our homemade luminescent measurement system was shown in Figure 1. This measurement system was composed of a photomultiplier tube (PMT), a photon counting unit (H8259-01, Hamamatsu) and a dark box. The black box could block outside light and had a transparent glass reaction tube (5.5 cm×1.2 cm) with a capacity of 5 mL volume . The reaction tube was straightly fixed to the front window of a PMT tube . The optimized detection wavelength of the PMT corresponded to the chemiluminescence emission wavelength (430 nm) of NSP-DMAE-NHS. A peristaltic pump was used to inject trigger solution into the reaction tube. The chemiluminescent emission intensity from the analyte in the glass tube was counted and transferred. The chemiluminescent signals were monitored and handled in real time by computer.

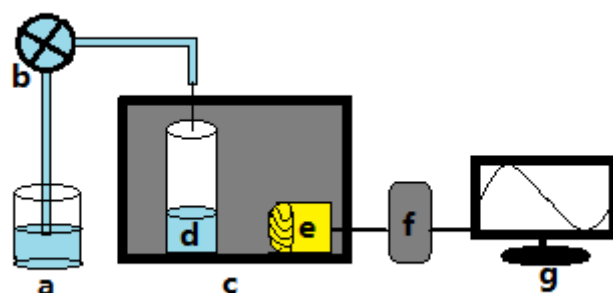


Figure 1. Luminescent measurement system (a) Substrate solution (b) Peristaltic pump (c) Black box (d) Reaction tube (e) Photomultiplier tube (f) Photon counting unit (g) Computer

### 2.3 Synthesis of Fe<sub>3</sub>O<sub>4</sub>magnetite nanoparticles

Fe<sub>3</sub>O<sub>4</sub> Magnetic nanoparticles (Fe-MNPs) were prepared by solvothermal synthesis reaction

with some modifications. Typically,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (1.35 g) was first dissolved in 40 mL ethylene glycol to form a clear solution, followed by addition of polyethylene glycol (1.0 g), afterward, sodium acetate (3.6 g) was added with stirring. The mixture was stirred vigorously for 30 min, and then sealed in autoclave (100 ml capacity). The autoclave was heated to  $200^\circ\text{C}$  for 10 h, and allowed to cool to room temperature. The black products were washed three times with deionized water and three times with ethanol respectively, and dried in a vacuum oven at  $80^\circ\text{C}$ .

#### 2.4 Synthesis of Amino-Functionalized $\text{Fe}_3\text{O}_4@\text{SiO}_2$ magnetite nanoparticles

The above prepared Fe-MNPs were stabilized by coated silica on the surface according to the stober process. Briefly, Fe-MNPs (340 mg) were dispersed in solvent that contained 250 mL ethanol and 30 mL deionized water under ultrasonic vibration for five minutes. Then 3 mL ammonia (28 wt.%) and 2 mL TEOS were added slowly with string and the reaction was continuous stirred for another 15 hours. Stopping the reaction, we separated the  $\text{Fe}_3\text{O}_4@\text{SiO}_2$  with a magnet dumping the supernatant liquid. Then, we used APTES as a amination reagent.  $\text{Fe}_3\text{O}_4@\text{SiO}_2$  magnetite nanoparticles (150 mg) in suspension under ultrasonic vibration in 50 mL ethanol were added 100  $\mu\text{L}$  APTES, the mixture was allowed to react with vigorous stirring on a magnetic stirrer at room temperature for 48 hours. The solid products were collected via an eternal magnetic field, washed with deionized water and ethanol and re-dispersed in 100 mL water.

#### 2.5 Synthesis of $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{Au}$ magnetite nanoparticles

Gold colloids with the size of 30 nm were prepared by reduction of  $\text{HAuCl}_4$  with sodium citrate. Then, 30 mL of the amino-functionalized  $\text{Fe}_3\text{O}_4@\text{SiO}_2$  nanoparticles dispersion was added dropwise to 100 mL of the citrate-stabilized gold colloids. With stirring for 1 hour, the resulting product was separated magnetically and washed with water.



Figure 2 a SEM of  $\text{Fe}_3\text{O}_4$  b SEM of  $\text{Fe}_3\text{O}_4@\text{SiO}_2$  c SEM of  $\text{Fe}_3\text{O}_4@\text{SiO}_2@\text{Au}$

#### 2.6 Immobilization of CAP base and CAP succinate molecules via BSA on Au-MNPs

##### 2.6.1 Method I: Preparation of CAP base-BSA-Au-MNPs conjugates

Au-MNPs (100  $\mu\text{L}$ ) were modified with carboxyl groups by immersing Au-MNPs (50 nm, 50  $\text{mg mL}^{-1}$ ) in a solution of MUA (5 mM) in ethanol for 16 h at room temperature. Then the carboxyl-(Au-MNPs) were washed twice with MES buffer (25 mM, pH 5.12) and the nanoparticles were activated by incubation with 100  $\mu\text{L}$  of a 1:1 ratio mixture of 200 mM EDC (N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride) and 50 mM NHS (N-hydroxysulfosuccin-imide) in MES buffer at  $37^\circ\text{C}$  for 1 h. Then, the activated carboxyl-Au-MNPs were washed three times with PBS (0.01 M, pH 7.4) in a magnetic field and mixed with 100  $\mu\text{L}$  BSA (10  $\text{mg mL}^{-1}$  in distilled water) with gently stirring for 2 h. The obtained BSA- Au-MNPs conjugates were separated by magnet for standby use.

CAP bases were conjugated to (Au-MNPs)-BSA as following: The above obtained (Au-MNPs)-BSA were dissolved in 2 mL PBS (0.01 M, pH 7.4) with adding 4.6 mg of N-hydroxysuccinimide NHS) and 7.7 mg of 1-ethyl-3- (3-dimethylamino-propyl)- carbodiimide hydrochloride (EDC). The solution was stirred for 30 min at  $37^\circ\text{C}$ . 35 mg CAP bases were then dropped in it and stirred for 2 h. Finally, the CAP base-BSA-Au-MNPs conjugates were separated with magnet.

### 2.6.2 Method II: Preparation of CAP succinate-BSA-Au-MNPs conjugates

In method II, CAP succinate would be anchored on the amine terminals of BSA via its carboxyl groups. In order to leave the amine terminals of the BSA to bind with the carboxyl group of CAP succinate in following, we immobilized BSA on Au-MNPs via its carboxyl terminals. So, the surface of Au-MNPs should be amino functionalized. A volume of 100  $\mu$ L of Au-MNPs (40 nm, 5% (m/v)) was immersed into a solution of cysteamine in distilled water for 4 h at room temperature. Then the amino-(Au-MNPs) were washed twice with MES buffer (25 mM, pH 5.12) and redissolved in PBS buffer. A volume of 100  $\mu$ L BSA (10 mg mL<sup>-1</sup> in distilled water) was activated by incubation with 100  $\mu$ L of a 1:1 ratio mixture of 200 mM EDC and 50 mM NHS in MES buffer at 37 °C for 1 h. The activated BSA solution was added to the amino-(Au-MNPs) suspension and the mixture was incubated at 37 °C for 2 h. After washing thoroughly with PBS, we got the BSA-Au-MNPs conjugates.

Due to the absence of carboxyl groups in CAP, CAP succinates were selected to conjugate with BSA-Au-MNPs. CAP succinates (17 mg) were dissolved in 2 mL of dry DMF. 4.6 mg of N-hydroxysuccinimide (NHS) and 7.7 mg of 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide hydrochloride (EDC) were added into the solution. Then the BSA-Au-MNPs were mixed together with the above activated CAP succinate solution and the mixture was shaken for 2 h at 37 °C. Finally, the CAP succinate-BSA-Au-MNPs conjugates were separated with magnet for standby use.

### 2.7 Competitive chemiluminescence assays using CAP base and CAP succinate immobilized particles

100  $\mu$ L of target CAP succinate sodium solution ( $10^{-5}$ ,  $10^{-4}$ ,  $10^{-3}$ , 0.01, 0.1, 1, 10, 100 and 1000 ng/mL) and the optimized amount of NSP-DMAE-NHS labeled mAb solutions were mixed together and incubated at 37 °C for 1 h. Then the mixture reacted with 100  $\mu$ L of CAP base or CAP succinate immobilized BSA-Au-MNPs at 37 °C for 1 h with gentle shaking. After that, the particles were washed with PBS (0.01M, pH 7.4) in magnetic field. The pretrigger ( $\text{H}_2\text{O}_2 + \text{HNO}_3$ ) and trigger (NaOH) solutions were added to the particles in sequence by a peristaltic pump to induce chemiluminescence. The instantly emitted light was collected with the PMT tube and measured with a photon counter.

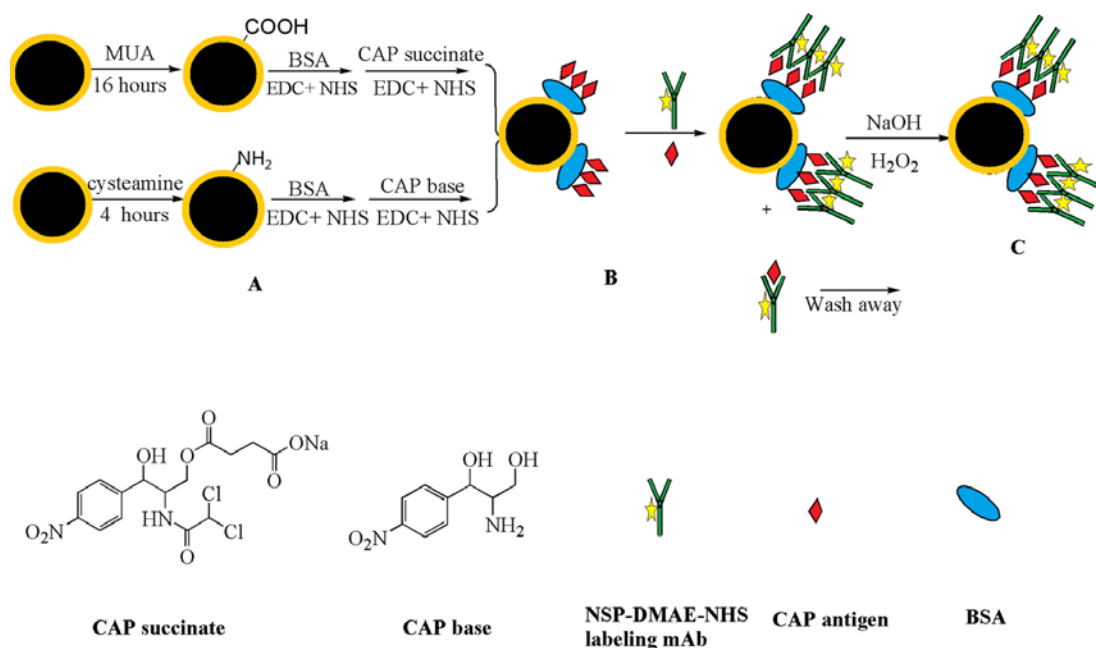


Figure 3 Flow chart of the CLIA reaction for CAP detection in competitive chemiluminescence assays. (A) Obtained CAP base/succinate-BSA-Au-MNPs conjugates; (B) Competitive immunoassay processes, using NSP-DMAE-NHS as a tracer; (C) Detection of chemiluminescence light intensity.

### 3. Results and discussion

Before construction of standard curves for different concentration of CAP, the amount of NSP-DMAE-NHS labeled mAb should be optimized. Different amount (5  $\mu\text{L}$ , 10  $\mu\text{L}$ , 20  $\mu\text{L}$ , 25  $\mu\text{L}$ , 30  $\mu\text{L}$ , 35  $\mu\text{L}$ , 40  $\mu\text{L}$ ) of NSP-DMAE-NHS labeled mAb ( $0.001\text{mg mL}^{-1}$ ) were bound on CAP immobilized BSA -Au-MNPs. Obviously, for both of the two methods the light intensities increased with the increase of the amount of NSP-DMAE-NHS labeled mAb in the range of 5-40  $\mu\text{L}$  and reached a saturation value at 35 $\mu\text{L}$ . In consideration of ensuring higher test sensitivity in the competitive reaction and tracer consumption, 30  $\mu\text{L}$  of NSP-DMAE-NHS labeled mAb was selected as the fixed volume for competitive reaction.

Different concentrations of CAP succinate sodium ( $10^{-5}$ ,  $10^{-4}$ , 0.001, 0.01, 0.1, 1, 10, 100 and 1000  $\text{ng mL}^{-1}$ ) were mixed with 30  $\mu\text{L}$  NSP-DMAE-NHS labeled mAb solution and the mixture were incubated at 37  $^{\circ}\text{C}$  for 1 h, and then 100  $\mu\text{L}$  of the CAP-BSA -Au-MNPs were added to competitively react with the NSP-DMAE-NHS labeled mAb. The right side of Figure 4 showed the calibration curves of CAP succinate sodium detection, where the peak heights of the chemiluminescence intensity were plotted against the logarithm of the concentrations of CAP succinate sodium. The limit of detection (LOD), which was calculated according to  $\text{LOD}=3s/m$  ( $s$  is the standard deviation of the peak light intensity of the lowest concentration of the linearity range and  $m$  is the slope of linear fitting curve), was 0.07  $\text{ng mL}^{-1}$  for Method I and 0.06  $\text{pg mL}^{-1}$  for Method II. The  $\text{IC}_{50}$  value, which is the competitor concentration that causes 50% growth inhibition, was calculated to be 0.086  $\text{ng mL}^{-1}$  for Method I and 0.14  $\text{ng mL}^{-1}$  for Method II. Those results shown much higher sensitivity than that of conventional ELISA methods. It might be greatly contributed by the increased binding ratio of the antibodies and antigens on the Au-MNPs and the high emitting efficiency of NSP-DMAE-NHS.

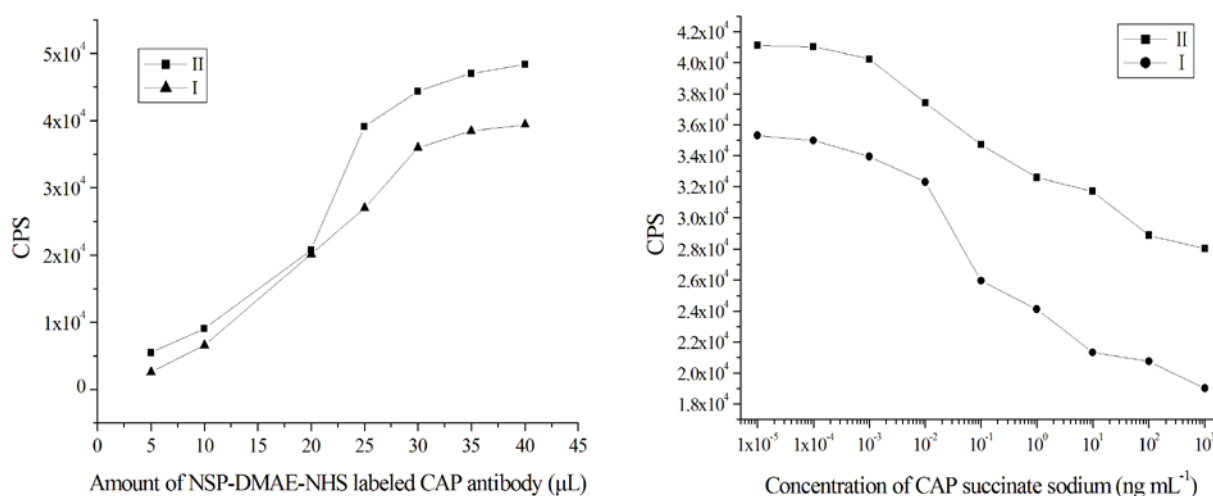


Figure 4 The left image is the emission intensity versus the amount of NSP-DMAE-NHS labeled mAb, Measurements were done in duplicate and the average values were taken. While the right side is the Calibration curves for detection of CAP succinate sodium.

### 4. Conclusion

In this research, we have developed a competitive CLIA using NSP-DMAE-NHS as luminescence marker and Au-MNPs as solid-phase carrier. A new strategy for immobilizing CAP molecules on Au-MNPs were exploited. That is immobilizing BSA on Au-MNPs at first and conjugating CAP molecules to the BSA afterward. The new strategy improved the immobilization efficiency because it need no prior synthesis of CAP-BSA conjugate and avoided dialysis in conventional preparation of CAP-BSA conjugate. Two calibration curves for CAP succinate sodium as target analyte were obtained. The LOD and  $\text{IC}_{50}$  values of Method I is 0.07  $\text{pg mL}^{-1}$  and 0.086  $\text{ng}$

mL<sup>-1</sup> respectively. The LOD and IC<sub>50</sub> values of Method II is 0.06 pg mL<sup>-1</sup> and 0.14 ng mL<sup>-1</sup> respectively. The above results demonstrated that the our method was much more sensitive than ELISA and luminol based chemiluminescence methods because of strong emission of chemiluminescence light of NSP-DMAE-NHS and usage of Au-MNPs particles served as the solid support. Therefore, the supposed strategy could be a potential alternative screening method to detect CAP and other antibiotics.

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