Direct Competitive Chemiluminescence Immunoassays Based on Gold Magnetic for Ultrasensitive Detection of Chloramphenicol

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Abstract. Chemiluminescent immunoassays (CLIA) based on gold magnetic nanoparticles (Au-MNPs) were developed for rapid analysis of chloramphenicol (CAP) in milk sample. Anti-CAP antibodies were immobilized on the surfaces of Au-MNPs, luminol (Method I) and 2',6'-DiMethylcarbonylphenyl-10-sulfopropylacridinium-9-carboxylate 4'-NHS Ester, (NSP-DMAE-NHS, Method II) were exploited in competitive CLIA for CAP detection in milk using a homemade luminescent measurement system. The sensitivities and limits of detection (LODs) of the two methods were obtained according to the inhibition curves. It indicated that NSP-DMAE-NHS as luminescence reagent (reaction II) was more sensitive and effective than luminol (reaction I). The LOD of reaction II reached 0.008 ng/ml while it was 4 ng/ml in reaction I. Moreover, the linear range of the inhibition curve of the former was wider than that of the latter. Such results indicated that the proposed CLIA stratery employing NSP-DMAE-NHS was more sensitive than other immunoassay method for CAP detection.

Keywords: *Chloramphenicol;* Chemiluminescence; immunoassays*; gold magnetic*

I. Introduction

Chloramphenicol (CAP), a kind of cheap and effective bacteriostatic broad-spectrum antibiotics, was widely used in animal husbandry to treat kinds of sensitive bacteria infection. However, it was found that CAP had serious adverse effects against human bodies, leading to bone marrow depression and even aplastic anemia [1-2]. Due to these side effects, it was banned from use in animal feeds and food in the European Union (EU), Switzerland, USA and other countries [3-4]. In 2002, the concept of Minimum Required Performance Limit (MRPL) was introduced by EU and it revised the technical criteria applied in the screening and confirmatory analysis of veterinary drug residues in food of animal origin [5]. The MPRL of CAP in milk, egg, meat, shrimp, honey and other animal food was set to be 0.3 ug/kg in EU [6]. The U.S. Food and Drug Administration

(FDA) had been researching more sensitive methods to reduce the detection limit from 0.3 ppb to 0.1 ppb. Even though, CAP is still illegally abused in animal farming because of its accessibility and low cost. Therefore, sensitive and effective detection methods are needed to screen this antibiotic. Various methods have been developed for detection of CAP residues in animal food. Conventional methods like microbiological methods [7] were not sensitive for CAP and were totally obsolete. Gas/liquid chromatography (GC/LC) [8-9] and gas/liquid chromatography-mass spectrometry (GC-MS/LC-MS) [10-12] are internationally recognized but expensive, complicated to operate, and not suitable for detecting lots of samples. Therefore, it is strongly required to develop simple 、 rapid and sensitive detection methods for CAP detection. Immunoassays including radio immunoassay (RIA) [13], enzyme-linked immunosorbent assay (ELISA) [14-15] have been reported frequently to screen CAP. RIAs have high sensitivity but exists radioactive waste, and ELISAs have shortcoming of higher limit of detection (LOD). . Chemiluminescent immunoassays (CLIAs) have some features such as

non-radioactive wastes, simple instrumentation, the very low limit of detection (LOD), high sensitivity, wide dynamic range and rapid analysis time in only several seconds. Those characteristics make it broadly used in clinical, environment, food and factory analysis in recent years [16-17].

In recent years, magnetic nanoparticles had been widely used as solid carry in RIA, ELISA and CLIA, due to: (i) the easier manipulation because the magnetic nanoparticles can be controlled in magnetic field instead of centrifuging repeatedly, (ii) the increased surface area as well as (iii) the faster assay kinetics because the nanoparticles are in suspension [18]. In addition, due to the improved washing and separation steps, the matrix effect is minimized in complex samples [19]. Gold magnetic nanoparticles (Au-MNPs), a kind of composite particle, have outstanding characteristics with combining the superparamagnetism of magnetic nanoparticles and the ability of being modified by biomolecules on gold surface. In addition, Au-MNPs are excellent biological markers because they are capable of conjugating with biomolecules, such as proteins and DNA, without hindering their biochemical activity [20]. It was reported that the sensitivity of immunomagnetic assay based on AuNPs was 10 times compared to that of immunomagnetic assay without AuNPs in detecting CAP [21].

Chemiluminescence reactions which exploited luminol (or N-(4-Aminobutyl)-N-ethylisoluminol, ABEI) and an enhancer (such as p-iodophenol) by hydrogen peroxide (H_2O_2) in the presence of horseradish peroxidase (HRP) were reported widely [22]. Currently, commercial system based on chemiluminescence in the market is few. A commercial system based on chemiluminescence exists and is commercialized by Randox (UK) (the Evidence Investigator system). However, Luminol, isoluminol and N-(4-Aminobutyl)- N-ethylisoluminol (ABEI) are kinds of old and widely used luminescence reagents, and are generally employed in CLIA with the presence of catalyst and enhancer which inevitably induce the enhancement of background light and the decrement of sensitivity. Weeks [23] synthesized acridinium esters (AE)-NHS [4-(2-Succinimidyfoxycarbonylethyl) phenyl-10methylacridinium -9-carboxylate fluorosulfonate] for the first time in 1983 and it has high luminous efficiency with no need of catalyst and enhancer. However, the thermal stability of AE-NHS is not so good that many researches have been done to syntheses more stable acridinium esters. NSP-DMAE-NHS (2',6'-DiMethylcarbonylphenyl -10-sulfopropylacridinium-9- carboxylate 4'-NHS Ester) is an ideal chemical luminescence immunoassay marker, of which the thermal stability and hydrolytic stability are both better than AE-NHS. Until now, there is no report about using NSP-DMAE-NHS as luminescence reagent to develop CLIA for CAP rapid analysis. This paper explored the Au-MNPs-based CLIA methods for CAP detection in milk samples respectively with luminol (reaction I) and NSP-DMAE-NHS (reaction II) as luminescence reagent.

II. Materials and methods

A. Synthesis of $Fe_3O_4@SiO_2@Au$ magnetite nanoparticles

Fe_3O_4 Magnetic nanoparticles (Fe-MNPs) were prepared by solvothermal synthesis reaction with some modifications. Typically, $FeCl_3 \cdot 6H_2O$ (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℃ for 10 h, and allowed to room temperature. The black products were washed and dried in a vacuum oven at 80℃.

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 250mL 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 strring and the reaction was continuous stirred for another 15 hours. Stopping the reaction, we separated the $Fe_3O_4@SiO_2$ with a magnet dumping the supernatant liquid. Then, we used APTES as a amination reagent. $Fe_3O_4@SiO_2$ magnetite nanoparticles (150mg) in suspension under ultrasonic vibration in 50ml ethanol were added 100 ul 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.

Gold colloids with the size of 30 nm were prepared by reduction of $HAuCl_4$ with sodium citrate. Then, 30 mL of the amino-functionalized $Fe_3O_4@SiO_2$ 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.
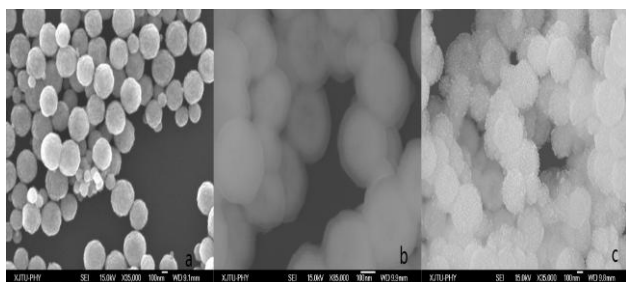
Figure 1. a SEM of Fe_3O_4 b SEM of $Fe_3O_4@SiO_2$ c SEM of $Fe_3O_4@SiO_2@Au$

B. Construction of luminescent measurement system

The diagram of our homemade luminescent measurement system was shown in Figure 2. This measurement system was composed of a photomultiplier tube (PMT), a photon counting unit (H8259-01, Hamamatsu) and a dark box. The counting unit was linked to a computer. The dark box was homemade which could block outside light and have a transparent glass tube (5.5cm×1.2cm) in it with 5ml capacity . The reaction tube was fixed to the correct position of an upright straight slot inside the box and the photocathode of a PMT tube was placed in front of it to receive the emitted light. The optimized detection wavelength of the PMT is corresponded to the chemiluminescence emission wavelength (430 nm). A capillary tubing was connected to the reaction tube through a needle in the upper part of the dark box, with the inlet part of it connected to an injector and a peristaltic pump for pumping trigger solution into the tube. The chemiluminescent emission intensity from the analyte in the glass tube was counted and transferred to computer. The chemiluminescent signals were monitored and handled in real time by computer.
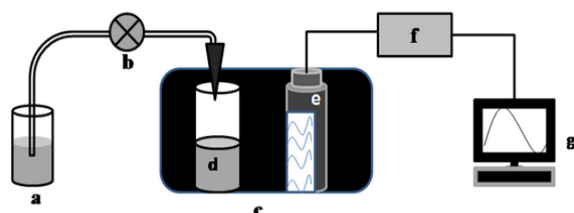
Figure.2. Luminescent measurement system (a) Substrate solution (b) Peristaltic pump (c) Black box (d) Reaction tube (e) Photomultiplier tube (f) Photon counting unit (g) Computer

C. Principle of the assay

Direct competitive assay method was employed. For reactions I, CAP-HRP conjugations were prepared. For reaction II, CAP was conjugated with the NSP-DMAE-NHS pre-labeled rabbit IgG protein. CAP is a kind of hapten with small molecule. It is hard to directly conjugate NSP-DMAE-NHS with CAP. Rabbit IgG，as a carrier protein, is used here for conjugating CAP and NSP-DMAE-NHS together to form a NSP-DMAE-NHS labeled CAP . Such labeled conjugations were competed with CAP standard solution to combine with the anti-CAP antibodies immobilized on the surface of Au-MNPs. A luminescent measurement system was constructed for the detection of chemiluminescent reactions. The sensitivity of both reactions were investigated and compared.

D. Preparation of tracers used in direct competitive assays

1)Preparation of CAP-HRP conjugates

10mg of CAP succinate were dissolved in 2ml DMF at room temperature and refrigerated at 4^oC for 10min, followed by the addition of Tributylamine (50 µl) and Isobutyl chloroformate (30 µl). The resulting mixture was incubated with stirring for 30min. 5mg of HRP were dissolved in 6ml of DMF (50%). The solution was stirred at $4\ ^oC$ and was adjusted to pH 8.0 with 1M NaOH. Next, the activated CAP succinate prepared above was added very slowly to the HRP solution and the mixture was incubated with magnetic stirring at room temperature for 2 hours. Subsequently, the resulting mixture was exhaustively dialyzed against PBS (0.01M, pH7.4) for 5 days at $4\ ^oC$ with changing PBS twice a day. The CAP-HRP conjugate thus prepared was utilized in the following study after appropriate dilution.

2) Preparation of CAP-(rabbit IgG)-(NSP-DMAE-NHS) conjugates

Preparation of NSP-DMAE-NHS -labeled rabbit IgG: Purified rabbit IgG (0.1 mg dissolved in 100 µl of carbonate buffer solution, 0.1M, pH 10.1) was labeled with 20 ug of NSP-DMAE-NHS dissolved in 10 µl of DMF. The mixture was gently stirred at room temperature for 24 hours, followed by the addition of 10 µl of L-lysine (10 mg/ml of distilled water) for 10min. The labeled rabbit IgG was isolated from unbound NSP-DMAE-NHS by gel filtration on a Sephadex G-50 column(1×25 cm) equilibrated with PBS (0.1M, pH6.3, 0.9% NaCl). The eluted solution was collected in dozens of bottles and detected by a UV spectrometer at 280 nm. Besides, the chemiluminescent activity of the eluted solution was measured. The part of high absorbance and also high chemiluminescent intensity was collected and frozen in single-dose vials. The procedure was according to a previous report with a partial modification [24].

CAP (20 mg) was dissolved in absolute ethyl alcohol (300 µl) and the solution was adjusted to pH

1 with 1M HCl. Then zinc powder (25 mg) was added and the solution was heated at 80 ^{o}C for 40 min. After that, the yellow supernatant was extracted by centrifuging to get amino-CAP. Thus, NH$_2$ group from the amino-CAP could react with COOH group of rabbit IgG (pre-labeled with NSP-DMAE-NHS). The conjugation was formed according to a previous report with a partial modification. As a result, NSP-DMAE-NHS was conjugated with CAP via rabbit IgG as a luminous marker.

E. Anti-CAP antibody immobilization on Au-MNPs

The immobilization process of the anti-CAP antibodies on the Au-MNPs is shown in Fig.4(A). Carboxyl-(Au-MNPs) which was formed by immersing Au-MNPs in a solution of 11-Mercaptoundecanoic acid (MUA, 5 mM) in ethanol for 16h at room temperature was washed twice with MES (2-(N-Morpholino) ethanesulfonic acid) buffer (25 mM, pH5.12). Afterwards, the carboxyl-(Au-MNPs) was re-suspended in a volume of 1 ml of MES buffer and then the carboxyl groups on the spheres were activated by incubation with 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 for 30 min. After washed three times with MES buffer and distilled water respectively, 100 μl of the Au-MNPs were incubated with 100 μl of anti-CAP antibody at 37 ^{o}C for 2 hours. After incubation, the supernatant was removed and washed three times with PBS (0.01M, pH7.4) in a magnetic field. In order to block the residual unreacted carboxyl groups, 300 μl of ethanolamine solution was added to prevent non-specific absorption. After blocking at 37 ^{o}C for 30 min, the Au-MNPs with anti-CAP antibodies immobilized on the surface were washed and stored in PBS at 4 ^{o}C for standby.

F. Determination of the tracers' optimized amount

Before construction of standard curves for different concentration of CAP in two chemiluminescence reactions, the optimized amount of tracers (CAP-HRP and CAP-(rabbit IgG)-(NSP-DMAE-NHS) conjugates) could be determined by binding them on the antibody-immobilized Au-MNPs with different concentrations. Fig.3 (A) shows the emission intensity (CPS) of the chemiluminescence corresponding to different amount of CAP-HRP. The concentration of CAP-HRP was 0.6 mg/ml. It can be seen that the chemiluminescence intensity increased with the increase of CAP-HRP amount in the range of 50-200 μl and the CPS reached saturation at 200 μl. However, it is interesting to note that signals showed a drop beyond 200 μl. This

extraordinary result may be explained by steric effect. Simply, the steric effect could influence the reaction between the CAP-HRP and the antibodies on the Au-MNPs. Once the amount of CAP-HRP was beyond 200 μl, the mass of CAP-HRP affected each other to generate conformational changes, which was unfavorable to make the antibodies recognize antigen. In this case, CAP-HRP saturation amount was 200 μl. In consideration of ensuring higher test sensitivity in the competitive reaction and tracer consumption, the optimal CAP-HRP amount of 180 μl was selected as the fixed volume for competitive reaction.
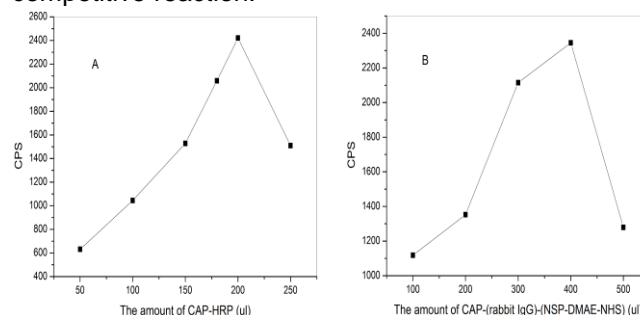
Figure.3. (A) The emission intensity versus the amount of CAP-HRP, of which the concentration was 0.6 mg/ml. Measurements were done in duplicate and the average values were taken. (B) The emission intensity versus the amount of CAP-(rabbit IgG)-(NSP-DMAE-NHS), of which the concentration was 0.01 mg/ml. Measurements were done in duplicate and the average values were taken.

Another competition tracer, CAP-(rabbit IgG)-(NSP-DMAE-NHS), was also investigated to determine an optimized amount in competition reaction in later experiments. Fig.3 (B) shows the CPS according to different amount of CAP-(rabbit IgG)-(NSP-DMAE-NHS), of which the concentration was 0.01 mg/ml. There also existed the steric effect and the saturation amount of this tracer was 400 μl. In consideration of the same reason, the optimal amount of CAP-(rabbit IgG)-(NSP-DMAE-NHS) of 300 μl was determined for competitive reaction in target CAP measurement.

G. Establishment of CLIA method based on Au-MNPs for CAP detection

1) Chemiluminescence reaction I: HRP/H$_2$O$_2$/ p-iodophenol/luminol

The process of reaction I is shown in Fig.4 (B). CAP succinate sodium and commercial milk powder were dissolved in deionized water to prepare the standard milk samples in which the concentration of the milk powder was 1mg/ml and the concentrations of the CAP succinate sodium were 0.01, 0.1, 1, 10, 100 and 1000 ng/ml respectively. The immobilized anti-CAP antibodies on Au-MNPs, which were prepared by the procedures described in the

previous section, were allowed to react with a mixture of certain amount of CAP-HRP tracer and the spiked milk samples. The mixture was allowed to incubate at 37 °C for 2 hours with gentle shaking. Then the Au-MNPs were washed with PBS in magnetic field. In this study, the substrate solution was made of luminol (0.5 mM), p-iodophenol (0.7 mM) and H_2O_2 (14 mM) in Tris-HCl buffer (20 mM, pH8.6) according to a former experiment. A volume of 100 µl of the mixture of luminol and p-iodophenol (1:1) was added to the Au-MNPs, followed by the injection of 100 µl of H_2O_2 by a peristaltic pump to induce chemiluminescence of the antibody-bound CAP-HRP on the Au-MNPs. At the same time, the chemiluminescence emission was measured by the luminescent measurement system. Finally, the used Au-MNPs in the immunoreaction tube were removed by washing with plenty of PBS buffer. After that, new Au-MNPs were introduced into the immunoreaction tube for the next measurement.

2) Chemiluminescence reaction II: Alkaline H_2O_2/ NSP-DMAE-NHS

The process of reaction II is shown in Fig.4 (C). The preparation of spiked milk samples was the same as reaction I except that the concentrations of the CAP succinate sodium were 0.0001, 0.001, 0.01, 0.1, 1, 10 ng/ml respectively. The immobilized anti-CAP antibodies on Au-MNPs, which were prepared by the procedures described in the previous section, were allowed to react with a mixture of certain amount of CAP-(rabbit IgG)-(NSP-DMAE-NHS) conjugates and the spiked milk samples. The mixture was allowed to incubate at 37 °C for 2 hours with gentle shaking. Then the particles were washed by PBS in a magnetic field. Here, HNO_3+ H_2O_2 and NaOH acted as the pre-trigger and trigger respectively to induce chemiluminescence emission. A volume of 100 µl of the pre-trigger was previously added to the Au-MNPs and 100 µl of trigger was injected into the tube by a peristaltic pump to induce chemiluminescence of the antibody-bound conjugates of CAP-(rabbit IgG)-(NSP-DMAE-NHS) on Au-MNPs. At the same time, the chemiluminescence emission intensity was measured versus time by the luminescent measurement system.

H. Direct competitive assays for CAP detection

In reaction I, 100 µl of spiked milk samples were mixed with 180 µl of HRP-CAP solution (0.6 mg/ml) and competitively reacted with the anti-CAP antibodies immobilized on the Au-MNPs. In Fig.5, curve (1) showed the relationship of chemiluminescence intensity (CPS) versus the

logarithm scale of concentrations of CAP succinate sodium in the range of 10^{-2}-10^3 ng/ml.
In reaction II, 100 µl of spiked milk samples were mixed with 300 µl of CAP-(rabbit IgG)-(NSP-DMAE-NHS) solution (0.01 mg/ml) and competitively reacted with the anti-CAP antibodies immobilized on the Au-MNPs. The curve (2) in Fig.5 showed the CPS versus the logarithm scale of concentrations of CAP succinate sodium in the range of 10^{-4}-10 ng/ml.
In Fig.5, it can be seen that chemiluminescence intensities were linearly decreased according to the increases of CAP succinate sodium concentrations. That is due to the competitive immune binding of the analyte and tracer to the immobilized antibodies. The IC_{50} values (the competitor concentration that causes 50% growth inhibition) of reaction I and reaction II were about 30 ng/ml and 0.03 ng/ml. The LOD value of reaction I and reaction II were approximately as 4 ng/ml and 0.008 ng/ml. In view of IC_{50}, the sensitivity of reaction II was improved 1000-folds compared with that of reaction I. Moreover, the latter was not only advantageous in higher sensitivity but also in wider linear range than the former. It indicates that applying NSP-DMAE-NHS as the chemiluminescent reagent is more sensitive and has a much wider measurement range than luminol in the CLIA. These results demonstrated that Reaction II was much more sensitive for the detection of CAP than some previously reported methods such as ELISA (IC_{50} of 10.5 ng/ml and LOD of 0.2 ng/ml, [25]), the pretreatment-free immunochromatographic assay (LOD of 10 ng/ml, [26]) and the time-resolved fluoroimmunoassay (TRFIA) (LOD of 0.05 ng/g, [27]).
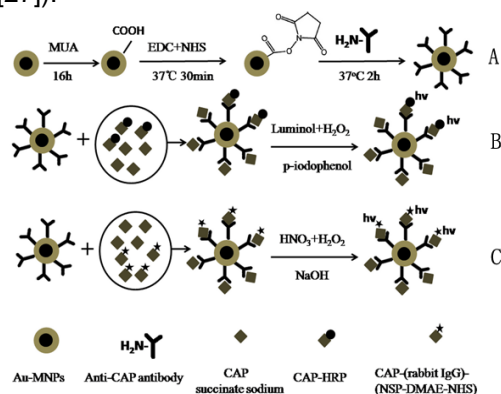

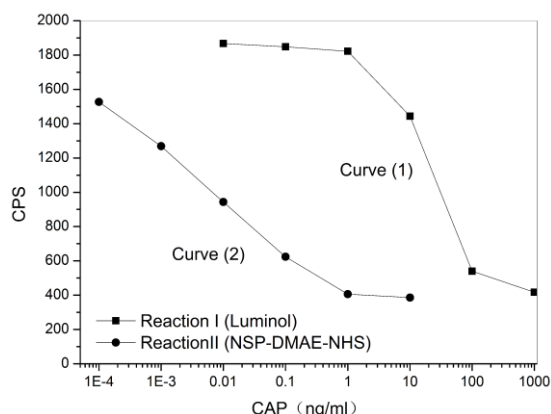**Figure.4**. two CLIA reactions for CAP detection.

Figure.5. Calibration curves for detection of CAP in milk

III. Conclusions

In conclusion, two competitive CLIA reactions using Au-MNPs as a solid-phase carrier and using NSP-DMAE-NHS as a new luminescence reagent were established for CAP detection. The Au-MNPs served as the solid support and provided a way of separation. Reaction II which used NSP-DMAE-NHS-labeled CAP-rabbit IgG as a tracer performed higher sensitivity and wider linear measurement range than reaction I which employed luminol with a tracer of CAP-HRP. As a new kind of luminescence reagent after luminol, NSP-DMAE-NHS showed excellent performance in applying to rapid CLIA detection of CAP. Based on this, We will carry on to apply our system and method to detect other residues in food, and to make this biosensor commercialized in the future.

IV. Acknowledgement Conclusions

V. References

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