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Employment of bromophenol red and bovine serum albumin as luminol signal co-enhancer in chemiluminescent detection of sequence-specific DNA

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ABSTRACT

Bromophenol red, known as chemical indicator, was found to act as a novel potent signal enhancer of the peroxidase-catalyzed luminol–H_2O_2 chemiluminescent (CL) reaction. It was found interestingly that bovine serum albumin (BSA) played a role in the enhanced chemiluminescent reaction (ECR). The addition of 2.5 mg mL^{-1} BSA into bromophenol red-enhance CL system showed 36 times stronger CL signal than that without addition of BSA. Mechanism study showed that the luminophors in the ECR were still 3-aminophthalate ion in an excited state (3-APA*). In addition, singlet oxygen ($^1O_2$) and hydroxyl radical ($^{\bullet}OH$) played a role in the ECR. The possible mechanism was discussed in the present study. The effect of pH, reaction time, and concentration of bromophenol red, BSA, luminol, and H_2O_2 on CL intensity of the peroxidase-catalyzed CL reaction was studied. The detection limit value (LOD) of HRP and streptavidin-modified HRP in the proposed ECR with bromophenol red and BSA was 0.20 ng mL^{-1} and 0.05 ng mL^{-1}, respectively. This novel luminol–H_2O_2–HRP–bromophenol red-BSA CL system was applied to the CL detection of sequence-specific DNA based on a magnetic separation process. As low as 0.4 fmol of target DNA could be sensitively detected using the proposed CL system without any amplification process. The obtained results demonstrate very promising perspectives for using bromophenol red and BSA to improve the sensitivity of CL detection of sequence-specific DNA. In addition, this novel ECR system can also be generalized for CL immunoassay, CL western blotting, and so on.

1. Introduction

Recently, a CL method is widely used in food analysis [1–6], medicine analysis [7], biological macromolecules detection [8] and immunoassay [9] because of its high sensitivity, wide dynamic range and cheap instruments [10,11]. Horseradish peroxidase (HRP) is a commonly used catalyst in luminol–H_2O_2 CL reaction, and has been widely used for the detection of hydrogen peroxide [12–14] and other analytes through coupled enzymatic reactions [15,16]. HRP catalyzes the oxidation reaction between luminol and H_2O_2 leading to the formation of a 3-aminophthalate ion (3-APA) in an excited state and subsequent emission of light when returning to the ground state [17].

Since the CL intensity of HRP-catalyzed luminol–H_2O_2 CL reaction is poor, certain compounds known as enhancers are added to the substrate mixture to augment CL intensity [18]. *P*-iodophenol (PIP) whose enhancement effect on HRP-catalyzed luminol CL reaction was discovered in 1988 [19] has become the most popular enhancer for luminol-based immunoassay, DNA hybridization assay, etc. [20–22]. The reported mechanism of ECR indicates that the enhancement effect of enhancer on CL may be attributed to the acceleration of the enzyme turnover by reaction of the enhancer with HRP compound II and the reversible electron-transfer reaction between the enhancer radical and luminol [23–25]. Although an effective enhancer it is, PIP is pretty smelly. Instead of PIP, a variety of substituted phenols such as 3-(10′-phenothiazinyl) propionic acid [17], 2-(4-hydroxyphenyl)-4,5-diphenylimidazole (HDI), 4-(4,5-diphenyl-1H-imidazol-2-yl) phenylboronic acid [14], and so on [26,27] have been applied as luminol signal enhancers.

In the present study, a novel enhancer bromophenol red (Fig. S1, Supporting Information) which is a chemical indicator was employed in HRP-catalyzed CL oxidation of luminol for the first time. Certain chemical indicators such as bromophenol blue and phenolphthalin have been reported as CL enhancer of HRP-catalyzed luminol CL reaction [28,29]. Bromophenol red is commonly used as photosensitizer or pH indicator in the past [30–32]. To the

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best of our knowledge, the application of bromophenol red as enhancer for HRP-catalyzed luminol–H_2O_2 CL system has not yet been reported. We also found interestingly that BSA played a role in the bromophenol red-enhanced CL system. Kapeliuch et al. reported a similar phenomenon in which BSA has a beneficial effect in the enhanced CL reaction in the presence of PIP [22]. In the present study, we investigated the enhancement effect of bromophenol red and BSA in luminol–H_2O_2–HRP CL system. The results exhibited that using bromophenol red and BSA as co-enhancer of luminol–H_2O_2–HRP CL system showed high sensitivity and good linearity.

To achieve sensitive detection of sequence-specific DNA, many analytical methods have been developed to improve detection sensitivity including using microsphere or liposome as signal amplification carrier [33], rolling cycle amplification based method [34], and so on. However, most of these amplification methods suffered from complex process of carrier preparation, time-consuming, and poor repeatability. Hence, development of simple and sensitive method for the detection of sequence-specific DNA is essential important. In the present study, the novel luminol–H_2O_2–HRP–bromophenol red-BSA CL system is sensitive for HRP which is a common label used in the detection of sequence-specific DNA. Hence, the proposed novel CL system was applied to the detection of hepatitis B virus (HBV) related sequence-specific DNA based on a magnetic separation process.

2. Experimental

2.1. Materials and chemicals

All chemicals and reagents were of analytical grade and used as received. Ultra pure water (18.2 MΩ cm^{-1}) was used throughout the current work. BSA was purchased from Bominda Biotech. Co. Ltd. (Tianjin, China). HRP was purchased from Aladdin Industrial Inc. (Shanghai, China). PIP and SA-HRP were obtained from Heowns Biochem. Technologies LLC. (Tianjin, China). Bromophenol red was obtained from Tokyo Chemical Industry Co., Ltd. (Shanghai, China). 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC · HCl) was acquired from J&K Scientific Ltd. (Beijing, China). Luminol was purchased from Alfa Aesar (Tianjin, China). Hydrogen peroxide (30%, v/v) and other chemical reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). The carboxyl terminated magnetic beads (Biomag® Plus Carboxyl, 1.5 μm) was obtained from Bangs Laboratories, Inc. All oligonucleotide probes were acquired from Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China) and had following sequences (Table S1, Supporting Information).

A stock solution of bromophenol red was prepared at a 0.4 M concentration in dimethyl formamide. Stock solution for 0.1 M luminol was made in 0.1 M NaOH solution. Working solutions of H_2O_2 were prepared fresh daily by dilution of 30% (v/v) H_2O_2. The stock solution for 1 mg mL^{-1} HRP was made in 10 mM PBS solution (pH 7.4).

2.2. Apparatus

CL measurements and CL spectra were performed with a BPCL chemiluminescence analyzer (Beijing, China) with a series of high-energy optical filters of 230, 260, 290, 320, 350, 380, 400, 425, 440, 460, 490, 535, 555, 575, 620, and 640 nm between the CL measuring cup holder and PMT.

2.3. Assay procedure for CL detection of HRP

For CL detection of HRP, 20 μL of reaction solution containing designed concentration of HRP, 2.5 mg mL^{-1} BSA, and 2 mM bromophenol red was mixed with 40 μL of 1 mM H_2O_2 in a 14×40 mm^2 glass tube at first. After standing at room temperature for 30 min, 50 μL of 1 mM luminol was injected into the glass tube immediately. At the same time, the light emission was measured by the BPCL chemiluminescence analyzer. The signal was imported to the computer for data acquisition ($n=3$).

2.4. Method validation for CL detection of HRP

To test the linearity and range for CL detection of HRP, 20 μL of reaction solution containing 0, 0.25, 0.5, 1, 1.5, 2, 2.5 ng mL^{-1} of HRP, 2.5 mg mL^{-1} BSA, and 2 mM bromophenol red was mixed with 40 μL of 1 mM H_2O_2 in a 14×40 mm^2 glass tube at first. The CL signals were then detected as described in "assay procedure for CL detection of HRP".

The precision of CL intensity for HRP detection was determinated by sequentially analyzing sets of several HRP standard solution of different concentration (0.5, 1.0, and 2.0 ng mL^{-1}). Each concentration level of HRP was measured seven times parallelly.

To evaluate the sepecificity of the proposed CL method for HRP detection, the effect of interferences including Na^+, Ag^+, NH_4^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Fe^{3+}, Fe^{2+}, Cl^-, SO_4^{2-} and $H_2PO_4^-$ on CL intensity was investigated, respectively. 20 μL of 1 ng mL^{-1} HRP, 2.5 mg mL^{-1} BSA, and 2 mM bromophenol red without interferent or with increasing concentration (from 1 μM to 1 mM) of interferent was mixed with 40 μL of 1 mM H_2O_2 in a 14×40 mm^2 glass tube at first. The CL signals were then detected as described in "assay procedure for CL detection of HRP".

2.5. Assay procedure for CL detection of sequence-specific DNA

21 μL of carboxyl terminated MBs were transferred into a centrifuge tube, where the beads were washed three times with 200 μL of imidazole buffer (0.1 M, pH 6.0) and re-suspended in 70 μL of 0.1 M imidazole buffer. The washed magnetic beads were divided equally into seven centrifuge tubes (10 μL per well), and then 90 μL of 0.1 M imidazole buffer containing 0.51 mg of EDC · HCl was added to each tube. The beads were activated at 37 °C with shaking for 20 min, and then mixed with 2.5 pmol of capture DNA. The mixture was incubated at 37 °C with shaking for 1 h, during which capture DNA was immobilized onto MBs surface. The resulting MB-cap DNA conjugates were washed one time with 100 μL of washing buffer (7 mM Tris buffer containing 0.05% Tween 20 and 0.1 M NaCl, pH 8.0), and then re-suspended in 100 μL of BA buffer (20 mM Tris buffer containing 0.5 M NaCl, pH 8.0) containing 1% BSA. The MB-cap DNA conjugates were blocked at 37 °C for 1 h. After washing three times with 200 μL of washing buffer, different amount of target DNA was added into each tube (final volume: 100 μL per well) and incubated at 37 °C for 1 h with shaking. After removing the solution, the beads were washed three times with 200 μL of washing buffer, and then reacted with 10 pmol of biotinylated reporter DNA at 37 °C for 1 h. The conjugates were washed three times with 200 μL of washing buffer. 100 μL of BA buffer containing 0.25 pmol SA-HRP and 0.5% BSA was added into each tube and incubated at 37 °C for 0.5 h, during which HRP was immobilized onto the conjugates via the specificity reaction between biotin and streptavidin. After washing three times with 200 μL of washing buffer, the magnetic-beads-DNA-HRP conjugates were re-suspended in 30 μL of ultrapure water and dissociated at 40 °C for 30 min. The resulting supernatant was isolated and mixed with 3 μL of 50 mg mL^{-1} BSA solution. Then, 10 μL of the mixture was transferred into a 14×40 mm^2 glass tube and analyzed as described above ($n=3$).