analytical chemistry

Article

pubs.acs.org/ac

Sensitive Chemiluminescence Immunoassay for *E. coli* O157:H7 Detection with Signal Dual-Amplification Using Glucose Oxidase and Laccase

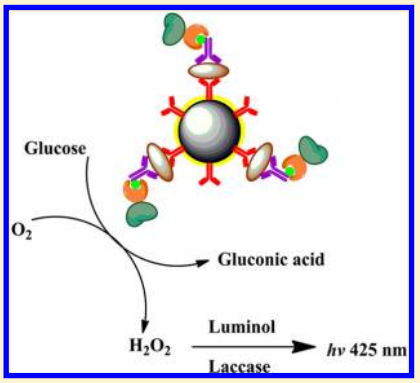
Yun Zhang,[†,‡,§] Chen Tan,[†,§] Ruihua Fei,[†,‡] Xiaoxiao Liu,[†,‡] Yuan Zhou,[†,‡] Jing Chen,[†] Huanchun Chen,[†] Rui Zhou,[†] and Yonggang Hu*[,†,‡]

[†]State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan 430070, China

[‡]College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

Supporting Information

ABSTRACT: A novel, sensitive chemiluminescence (CL) immunoassay for *Escherichia coli* O157:H7 detection with signal dual-amplification using glucose oxidase (GOx) and laccase was investigated. The method was based on the characterization of a luminol–H_2O_2–laccase reaction. Compared with the horseradish peroxidase-based biosensor, laccase exhibited high catalytic activity in strong alkaline medium, which was compatible with the luminol system. The capture antibody was immobilized onto the magnetic bead (MB) surfaces. The detection antibody was linked with GOx through biotin–avidin recognition. Accordingly, the bioconjugation of MB–caputure antibody–*E. coli* O157:H7–detection antibody–GOx catalyzed the substrate glucose, thereby generating H_2O_2. *E. coli* O157:H7 was then detected by measuring the CL intensity after H_2O_2 formation. Under optimal conditions, the calibration plot obtained for *E. coli* O157:H7 was approximately linear from 4.3×10^3 colony-forming unit (CFU) mL^{-1} to 4.3×10^5 CFU mL^{-1}, and the total assay time was <2.0 h without any enrichment. The limit of detection for the assay was 1.2×10^3 CFU mL^{-1} (3σ), which was considerably lower than that of enzyme-linked immunosorbent assay method (1.0×10^5 CFU mL^{-1}) (3σ). A series of repeatability measurements of using 1.7×10^4 CFU mL^{-1} *E. coli* O157:H7 exhibited reproducible results with a relative standard deviation (RSD) of 3.5% ($n = 11$). Moreover, the proposed method was successfully used to detect *E. coli* O157:H7 in synthetic samples (spring water, apple juice, and skim milk), which indicated its potential practical application. This protocol can be applied in various fields of study.

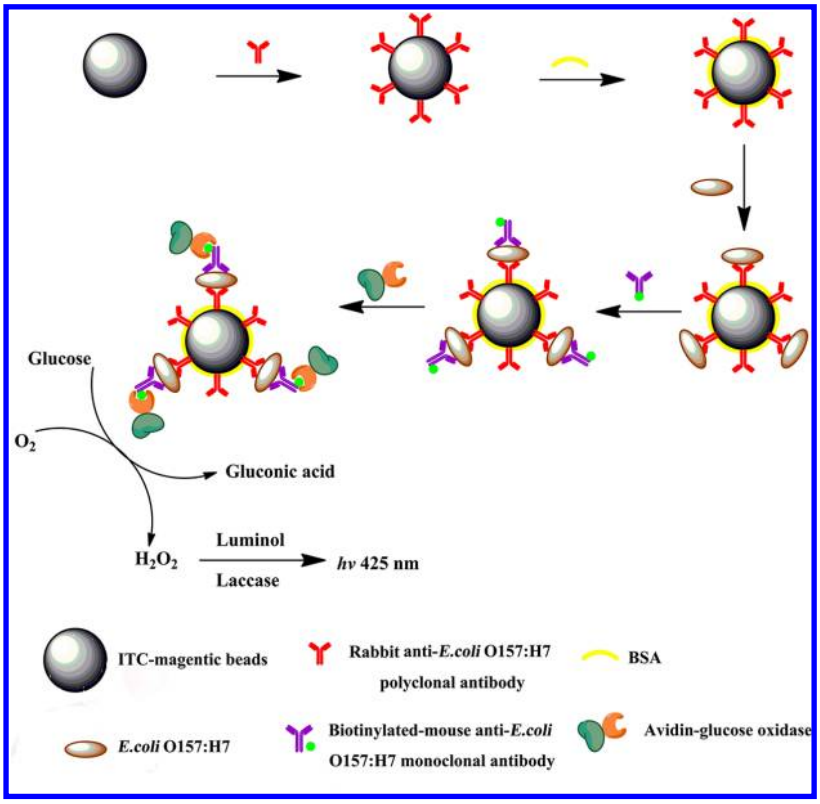


Pathogenic bacteria in food and pharmaceutical products, even at infinitesimal amounts, seriously affect on public health. The Centers for Disease Control estimates that one in six people in the United States are infected annually, with approximately 125000 people being hospitalized and 3000 cases resulting in untimely death.[1] Among the pathogenic bacteria, *Escherichia coli* O157:H7 is one of the most harmful food pathogens that can cause serious foodborne illnesses and deaths.[2,3] Therefore, developing new techniques for rapid and sensitive detection of *E. coli* O157:H7 is extremely important to prevent catastrophic outbreaks caused by public consumption. Conventional methods such as culturing is substantially time-consuming (i.e., 24 to 48 h), thereby delaying the results and making timely prevention of epidemic outbreaks difficult. Nonculture-based methods are being developed based on the principle of specific biomolecule recognition of antibody and nucleic acid. Nucleic acid-based tests such as real-time polymerase chain reaction (PCR)-based methods[4,5] have several advantages over conventional methods, including rapid results, low limits of detection (LODs), and high specificity. However, nucleic acid-based tests require expensive equipment and skilled technical staff and may not be appropriate for the developing countries. In addition, nucleic acid-based methods have the risk of releasing amplified nucleic acids into the environment, thereby contaminating the subsequent analysis. Immunoassays are based on the highly biospecific recognition interactions of antibody–antigen. Immunoassays are widely applied in pharmaceutical, toxicological, and environmental analyses, as well as bioanalysis and clinical chemistry because of their high sensitivity, high selectivity, rapid detection, and possible analysis of difficult matrices without extensive pretreatment. Immunoassay-based methods have been successfully developed for the detection of *E. coli* O157:H7, such as enzyme-linked immunosorbent assays (ELISAs),[6,7] electrochemical biosensor,[8,9] piezoelectric quartz crystal techniques,[10] fluorescence spectroscopy biosensor,[11,12] surface plasmon resonance,[13] and chemiluminescence (CL) biosensor.[14−16] Sandwich ELISA formats are commonly used to detect microorganisms. However, the conventional ELISAs are laborious, time-consuming, and insensitive to the matrix effect.

Received: September 9, 2013
Accepted: December 26, 2013
Published: December 26, 2013

dx.doi.org/10.1021/ac4028774 | *Anal. Chem.* 2014, 86, 1115−1122

Scheme 1. Schematic of the Laccase-Based CL Immunoassay for *E. coli* O157:H7 Detection (Not to Scale)



Conventional ELISAs also have low detection sensitivity because of the use of the colorimetric detection.

In recent years, studies have focused on magnetic bead (MB)-based CL immunoassays for the determination of bacteria[17,18] and other molecules (e.g., proteins, hormones, drugs, nucleic acids, and environmental pollutants).[19−21] MBs are an ideal platform candidate that enable quick and efficient purification from crude samples, thereby eliminating the need for most of the pretreatment steps and the matrix effects from samples.[22,23] The advantages of CL detection include sensitivity, rapidity, wide dynamic range, relatively simple equipment, and low instrumentation costs because no external light source or optics are needed.[24,25] On the basis of initial findings demonstrating the ability and efficacy of laccase to catalyze the luminol−H_2O_2 reaction, we introduced a novel MB-based CL immunoassay method in the present study. The proposed method was employed to detect *E. coli* O157:H7.

The schematic is depicted in Scheme 1. First, the capture antibody is immobilized on the surface of MBs. Samples infected with *E. coli* O157:H7 are then added to induce primary immunorecognition, followed by biotin-conjugated detection antibody to initiate the second immunorecognition event. Avidin-conjugated glucose oxidase (GOx) is attached on the surface of MBs through biotin−avidin recognition reaction to form immunocomplex-MBs (IMBs). Unbound GOx−antibody is washed out with a buffer solution after the immunological reaction. When the IMBs are exposed to a solution containing glucose, GOx catalyzes the conversion of glucose into gluconic acid and H_2O_2.[26,27] Finally, laccase and luminol are added to produce CL signals, and the *E. coli* O157:H7 is quantified through a CL assay of the solution. The proposed method introduces an approach to design CL immunosensors for detecting other significant analytical targets.

EXPERIMENTAL SECTION

Materials. Rabbit anti-*E. coli* O157:H7 polyclonal antibody, mouse anti-*E. coli* O157:H7 monoclonal antibody, and ELISA kit were purchased from Prajna Biology (Shanghai, China). Biotin *N*-hydroxysuccinimide ester and laccase (EC 1.10.3.2; p-diphenol:dioxygen oxidoreductases from *Trametes versicolor*) were purchased from Sigma-Aldrich Chemicals Company (St. Louis, MO). The monoclonal anti-O157 antibody was conjugated with (+)-biotin *N*-hydroxysuccinimide ester (Sigma-Aldrich), according to the manufacturer's instructions. Free biotin molecules in the conjugation reaction mixture were removed using a dialysis bag. Avidin-conjugated GOx was supplied by Rockland. Luminol (3-aminophthalhydrazide) was obtained from Alfa Aesar. Isothiocyanate-terminated MBs (ITC-MBs; 3 μm in diameter) were purchased from GoldMag Biotech (Xian, China). DL2000Maker, forward primer (5′ CGG ACA TCC ATG TGA TAT GG 3′), reverse primer (5′ TTG CCT ATG TAC AGC TAA TCC), dNTP, and Taq DNA polymerase were synthesized at Sangon Biotech (Shanghai, China). Bacterial gene DNA kit was purchased from Tiangen Biotech (Beijing, China). Working solutions of luminol were prepared by diluting the stock solution with ultrapure water. Stock solutions including 1.0 mg mL^{-1} polyclonal antibody, 0.1 mg mL^{-1} monoclonal anti-O157 antibody-conjugated biotin, and 4.0 mg mL^{-1} avidin-conjugated GOx were prepared by separately dissolving these proteins in 0.01 mol L^{-1} PBS (pH 7.4) with NaCl (0.14 mol L^{-1}) and KCl (2.7 mmol L^{-1}) solution. The stock solutions were stored at 4 °C. Luminol was dissolved in 1.0 mol L^{-1} NaOH, diluted to 32.0 mmol L^{-1}, stored at 4 °C, and used within 3 d of preparation. The *E. coli* O157:H7 (EDL933), *Salmonella* (CICC21497), *E. coli* DH5α, *ExPEC* 97 (CCTCCAB 2013346), *ExPEC* 80 (CCTCCAB 2013345), *Streptococcus*

dx.doi.org/10.1021/ac4028774 | *Anal. Chem.* 2014, 86, 1115−1122

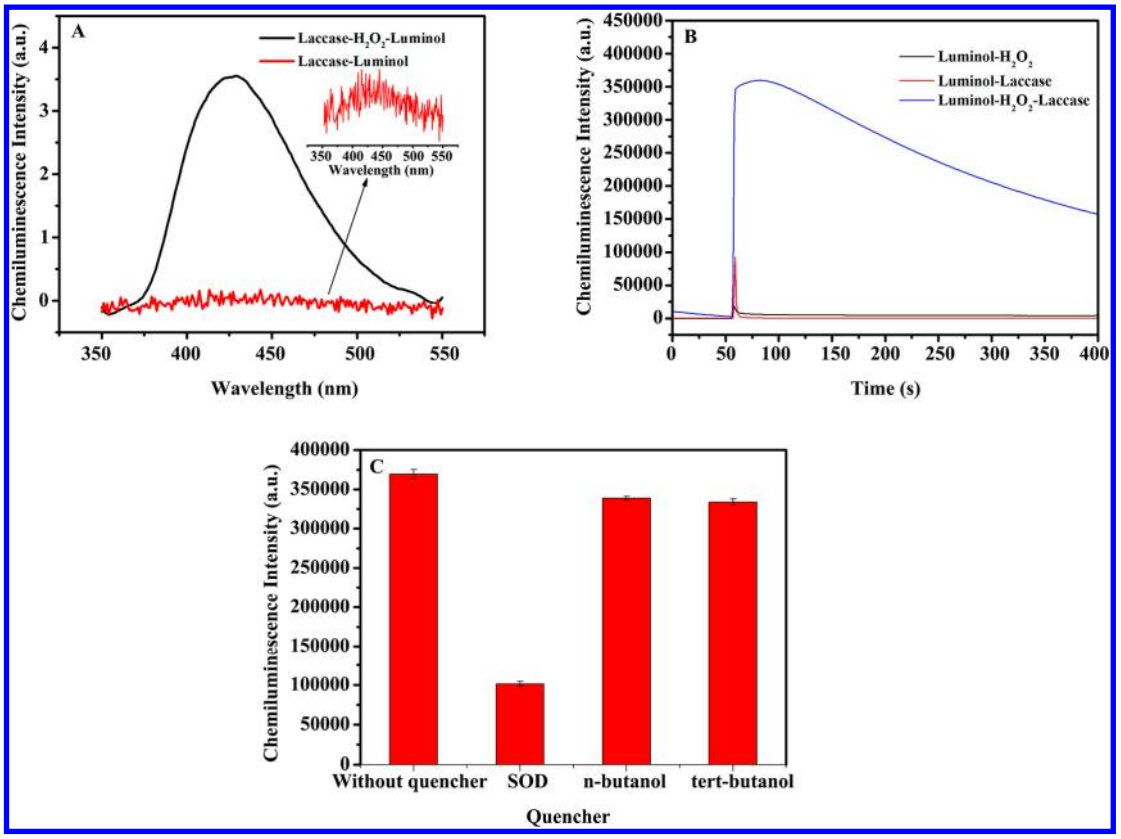


Figure 1. Characterization of the luminol–H_2O_2–laccase CL reaction. (A) CL spectra for the luminol–H_2O_2–laccase and luminol–laccase CL reactions. (B) Kinetic characteristics of the luminol–H_2O_2–laccase, luminol–H_2O_2, and luminol–laccase reactions. (C) Effects of quenchers on the luminol–H_2O_2–laccase CL intensity. Conditions: 0.1 mg mL^{-1} SOD, 0.1 mol L^{-1} *n*-butanol, 0.1 mol L^{-1} *tert*-butanol.

suis (SC19), *E. coli* O91 (CCTCCAB 2013344), *E. coli* O97 (CCTCCAB 2013342), and *E. coli* O149 (CCTCCAB 2013343) were provided by State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University. All other reagents were analytical grade and purchased from local commercial suppliers.

Apparatus. A Tecan Infinite F200 Multifunctional microplate reader (Shanghai, China) was used to measure CL signals. A 5334 PCR system was obtained from Eppendorf (Germany). An AlphaImager Mini Gel Imaging System was purchased from ProteinSimple (Silicon Valley). A Platform Constant Temperature (Shanghai Jing Hong Laboratory Instrument Company, Ltd., Shanghai, China) was used as a shaking incubator. The pH of all buffer solutions was measured using an Orion pH meter (United Initiators Company, Ltd., Shanghai, China). The kinetic characteristics of the CL reaction were analyzed using a BPCL-1-TIC ultraweak luminescence analyzer (Beijing Brothers Chuanghe Technology and Trade Company, Ltd., Beijing, China). Typical CL emission spectra were analyzed with a Shimadzu RF-5301PC fluorescence spectrophotometer (Tokyo, Japan). All solutions were prepared and diluted using ultrapure water (18.2 MΩ cm) produced by a Model Cascada IX laboratory ultrapure water system (Pall Company, Ltd.).

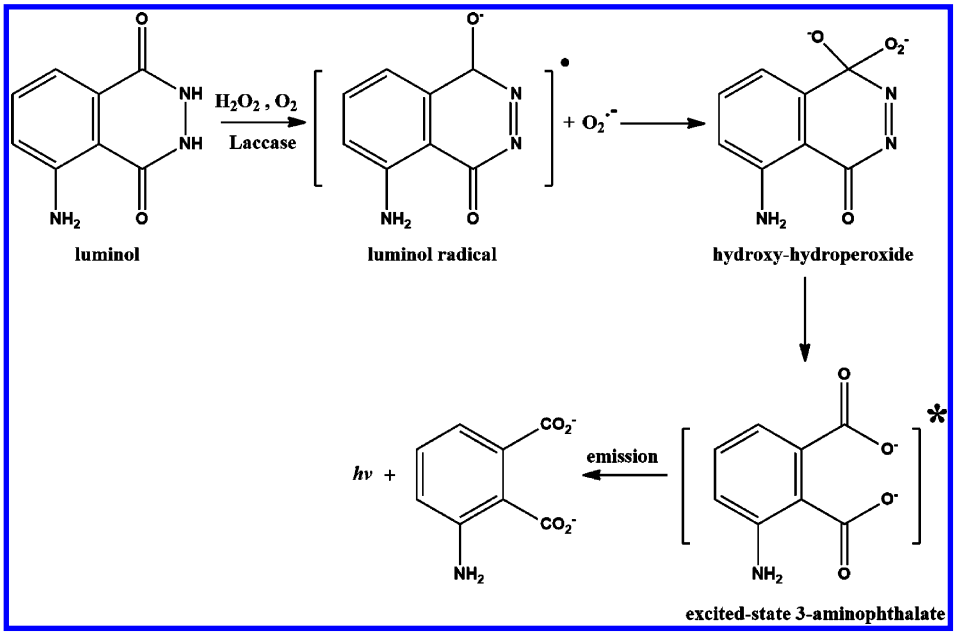
Laccase Purification. Laccase was purified according to the protocol described by Blanford with slight modifications.[28] In a typical procedure, 10.0 mg of laccase was dissolved in 1.0 mL of 10.0 mmol L^{-1} sodium acetate buffer (pH 5.5) and centrifuged using CR21G II high-speed refrigerated centrifuge (Hitachi Ltd., Japan) at 14000 rpm and 4 °C for 1 h. The supernatant was placed in a DEAE-SephadexA-50 (BioMan, Shanghai, China) anion-exchange column (previously equilibrated with buffer) at 4 °C. Laccase (isoform mixture) was eluted with 60.0 mmol L^{-1} ammonium sulfate in 20.0 mmol L^{-1} sodium acetate buffer (pH 5.5) and stored at 4 °C. Working solutions of laccase were prepared by diluting the stock solution with ultrapure water.

Bacterial Strains and Growth Conditions. *E. coli* O157:H7 strain (EDL933) was used in this study. The bacterial culture was routinely grown at 37 °C in Luria–Bertani medium for 12 h. Viable cell counts were determined by plating on MacConkey agar. After overnight incubation, the colonies were counted. Colony-forming units (CFUs) were counted as 1.72×10^9 CFU mL^{-1} in 0.01 mol L^{-1} PBS buffer (pH 7.4) containing 0.1% NaN_3, and the colonies were stored at 4 °C until use.

Preparation of Anti-O157:H7 IMBs. IMBs were prepared by coating the antibody specific to *E. coli* O157:H7 into superparamagnetic ITC-MBs according to the manufacturer's instructions, as described in our previous report.[29] In brief, 1 mg ITC-MBs was washed twice with 200 μL of PBS (pH 7.4) to balance the salt concentration, and the supernatant was discarded. Then, 200 μL of 1 mg mL^{-1} antibody was covalently attached onto the surface of ITC-MBs. The mixture of antibody and ITC-MBs was shaken (180 rpm) at 37 °C for 30 min. After discarding the supernatant, the IMBs were washed twice with 200 μL of PBS to remove the residual antibody. The antibody-coated IMBs were blocked in PBS containing 5% bovine serum albumin (BSA) and 15% skim milk at 37 °C for 2 h. The beads were separated using a magnet and resuspended in 0.01 mol

dx.doi.org/10.1021/ac4028774 | *Anal. Chem.* 2014, 86, 1115–1122

Scheme 2. Mechanism of Laccase Enhanced Luminol−H_2O_2 Chemiluminescence System



L^{-1} PBS (pH 7.4) containing 0.1% NaN_3 and 0.1% BSA. The IMBs were stored at 4 °C until use and can remain stable for more than 6 months.

Procedure for Measurement of CL Enzyme Immunoassay Analysis (CLEIA). The schematic of the MB-based CLEIA (MB-CLEIA) is shown in Scheme 1. Exactly 15.0 μL of antibody-coated MBs was mixed with 100.0 μL of *E. coli* O157:H7, and then 100.0 μL of biotin-labeled anti−*E. coli* O157:H7 detection antibody was added. All reactions were allowed to proceed at 37 °C for 30 min. Through biotin−avidin recognition, the MBs were exposed to 100 μL of avidin−GOx for 20 min. Then, 100 μL of 0.02 mol L^{-1} glucose in PBS solution (pH 7.4) was added. The mixture was incubated with shaking at 37 °C for 30 min. After magnetic separation, 100 μL of supernatant containing H_2O_2 was mixed with 50.0 μL of laccase and 50.0 μL of luminol. The pH of this mixture was then immediately adjusted to 11.0 using 0.1 mol L^{-1} NaOH, and the CL signals were measured using a multifunctional microplate reader.

PCR/Electrophoresis. *E. coli* O157:H7 DNA was detected using a 5334 PCR system (Eppendorf, Germany) in a 25.0 μL mixture containing 2.0 μL of *E. coli* bacterial DNA, 2.5 μL of 10× PCR buffer, 2.0 μL of dNTP, 0.5 μL of both forward and reverse primers, 0.5 μL of Taq DNA polymerase, and 17.0 μL of double-distilled water. The samples were subjected to 30 PCR cycles, each consisting of 5 min of initial denaturation at 95 °C, 30 s of denaturation at 95 °C, 40 s of annealing at 60 °C, and 30 s of elongation at 72 °C. The samples were stabilized at 72 °C for 8 min. Finally, 10.0 μL of PCR products were tested by electrophoresis on 0.8% agarose gels and photographed under UV light.

RESULTS AND DISCUSSION

Characterization of the Luminol−H_2O_2−laccase CL System. Laccase, which is a member of blue multicopper oxidases, is widely distributed among plants, insects, fungi, and bacteria.[30] Given their broad substrate range, laccases are useful in biotechnological applications, such as paper pulp bleaching, synthetic dye decolorization, bioremediation, biosensing, chemical synthesis, and immunoassay.[31,32] Study on laccase-based biosensors mainly focuses on the spectrophotometric and electrochemical methods. In the present work, we studied enhancement property of laccase in the CL reaction of luminol−H_2O_2. On the basis of the findings, a novel laccase-based CL sensor was then proposed for immunoassay.

The effects of laccase on the luminol−H_2O_2 CL system were investigated (Figure 1), and the reaction system of luminol−H_2O_2−laccase was extensively studied. The emission wavelength was observed at around 425 nm (Figure 1A), which indicated that the luminophore of the luminol−H_2O_2−laccase CL system was the excited-state 3-aminophthalate anion. Thus, laccase addition did not result in the generation of a new luminophore for this CL system. The enhanced CL signals were thereby ascribed to the possible catalysis from laccase because it containing Cu atoms in its catalytic center. The enhancement effect was possibly due to the Cu atoms in its catalytic center of laccase. The kinetic curve in Figure 1B shows that luminol oxidation by H_2O_2 generated weak CL in alkaline media. The CL emission of luminol−H_2O_2 immediately peaked after luminol injection and rapidly decreased under these analytical conditions. Thus, CL signal intensity could be significantly enhanced and increased immediately after lacasse injection into the mixture solution of luminol−H_2O_2. Moreover, the CL signal of luminol−laccase reaction without H_2O_2 was observed in our work. A similar emission wavelength at around 425 nm was also obtained. Given that laccases can oxidize a wide variety of organic and inorganic compounds including diphenols, polyphenols, diamines, and aromatic amines by reducing molecular oxygen to water,[33] we believe that the laccase can catalyze luminol oxidation to produce an excited-state 3-aminophthalate anion and a CL signal in the presence of molecular oxygen (O_2). The results in Figure 1B indicate that the kinetic curve of luminol−laccase in the presence of O_2 was similar to the luminol−H_2O_2 reaction. To determine the function of superoxide ion ($O_2^{\bullet-}$) and $HO^{\bullet}$ in the reaction, the effects of quencher superoxide dismutase (SOD) and two $HO^{\bullet}$ quenchers, namely, tert-butanol, and *n*-butanol were investigated (Figure 1C). The CL intensity was

dx.doi.org/10.1021/ac4028774 | *Anal. Chem.* 2014, 86, 1115−1122

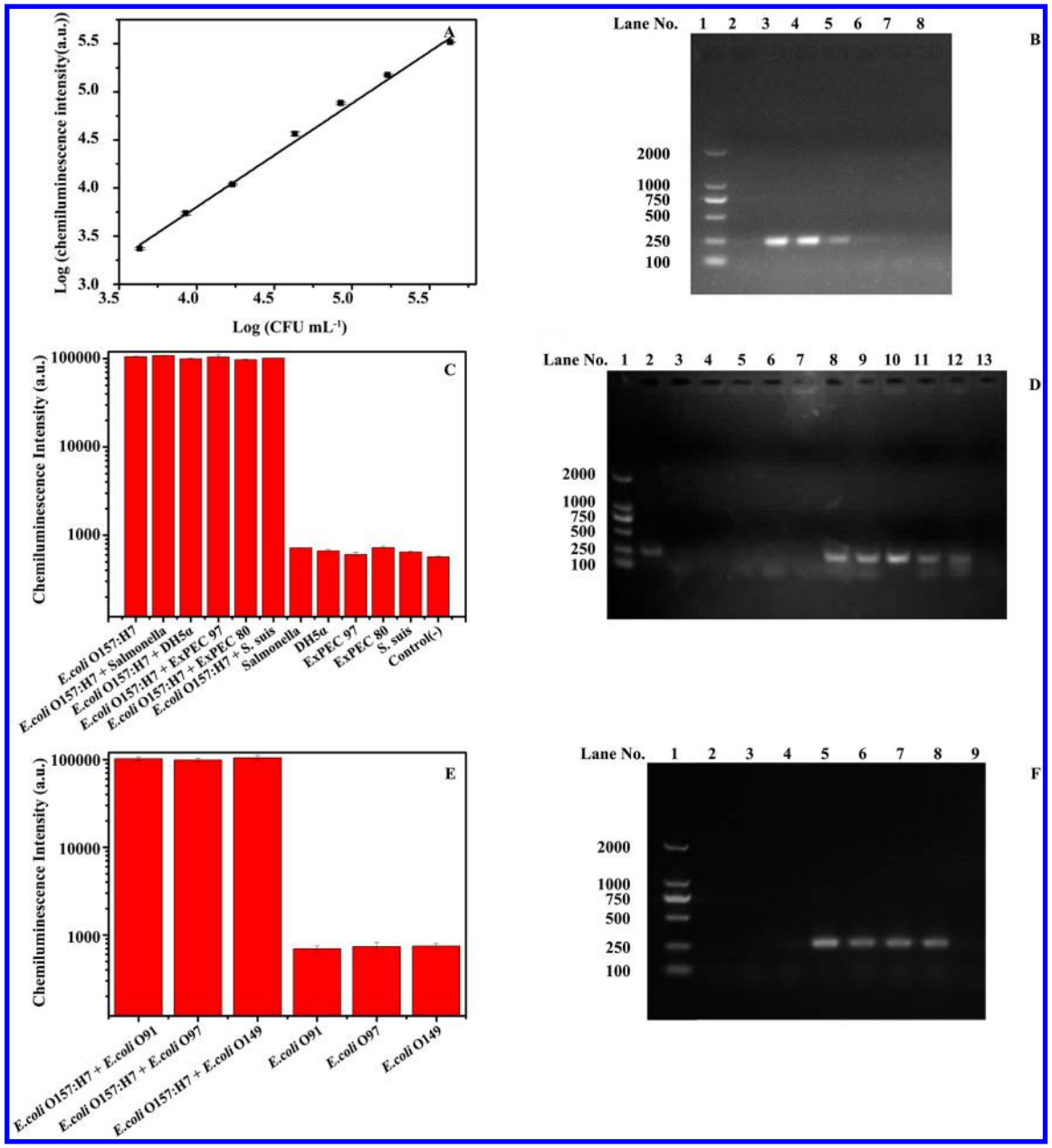


Figure 2. (A) Calibration curve for CL intensity along with the *E. coli* O157:H7 concentration in a log–log plot under the optimal conditions. (B) Agarose gel electrophoresis detection of *E. coli* O157:H7 DNA. Lane 1 is the molecular marker. Lane 2 is the negative control. Lanes 3 to 8 are 10-fold dilutions ranging from 4.0×10^6 CFU mL^{-1} to 4.0×10^1 CFU mL^{-1}. (C) Specificity study for the laccase-based CL immunoassay for *E. coli* O157:H7 detection. Red bars show the CL intensity (from left to right) for *E. coli* O157:H7 (1.0×10^5 CFU mL^{-1}); *Salmonella*, *E. coli* DH5*α*, *ExPEC 97*, *ExPEC* 80, and *S. suis* (1.0×10^5 CFU mL^{-1}) interfered with 1.0×10^5 CFU mL^{-1} *E. coli* O157:H7; 1.0×10^5 CFU mL^{-1} *Salmonella*, *E. coli* DH5*α*, *ExPEC 97*, *ExPEC 80*, *S. suis*, and negative control. (D) Agarose gel electrophoresis of PCR products: lane 1 is the molecular weight marker, whereas lane 13 is negative control. Lane 2, 1.0×10^5 CFU mL^{-1} *E. coli* O157:H7, lanes 3 to 7, *E. coli* DH5*α*, *Salmonella*, *ExPEC* 97, *ExPEC* 80, and *S. suis* (1×10^5 CFU mL^{-1}); lanes 8 to 12, *E. coli* DH5*α*, *Salmonella*, *ExPEC* 97, *ExPEC* 80, *S. suis* interfered with 1.0×10^5 CFU mL^{-1} *E. coli* O157:H7, respectively. (E) Specificity study for the laccase-based CL immunoassay for *E. coli* O157:H7 detection. Red bars show the CL intensity (from left to right) for *E. coli* O91, *E. coli* O97, and *E. coli* O149 (1.0×10^5 CFU mL^{-1}) interfered with 1.0×10^5 CFU mL^{-1} *E. coli* O157:H7; 1.0×10^5 CFU mL^{-1} *E. coli* O91, *E. coli* O97, and *E. coli* O149. (F) Agarose gel electrophoresis of PCR products: lane 1 is the molecular weight marker, whereas lane 9 is the negative control; Lane 5, 1.0×10^5 CFU mL^{-1} *E. coli* O157:H7; lanes 2 to 4, *E. coli* O91, *E. coli* O97, and *E. coli* O149 (1×10^5 CFU mL^{-1}); lanes 6 to 8, *E. coli* O91, *E. coli* O97, and *E. coli* O149 interfered with 1.0×10^5 CFU mL^{-1} *E. coli* O157:H7, respectively. Error bars indicate standard deviations from five independent measurements.

significantly inhibited by SOD and was almost unchanged by the introduction of the HO$^{\bullet}$ quenchers. Consequently, we assumed that $O_2^{\bullet-}$ was generated during the reaction without HO$^{\bullet}$. On the basis of the above results, the entire enhancement mechanism is deduced in Scheme 2. First, the luminol was oxidized to a luminol radical by H_2O_2 and dissolved oxygen (O_2). Meanwhile, a superoxide radical anion is expected to occur. The one was supposed to be generated from H_2O_2. Another possibility was that the oxygen dissolved in the solution reacted with luminol radicals to generate superoxide radical anions.[34] Further electron-transfer processes between luminol radicals and superoxide radical anions formed the key

dx.doi.org/10.1021/ac4028774 | *Anal. Chem.* 2014, 86, 1115–1122

intermediate alpha-hydroxy-hydroperoxide.[35] Then, the hydroxy-hydroperoxide decomposed to excited-state 3-aminophthalate anions, leading to the emission of CL upon energy dissipation of 3-aminophthalate ions from the excited to the ground state. This decomposition reaction was supposed to be the rate-determining step of luminol CL.[36] However, the reaction of luminol with hydrogen peroxide and dissolved oxygen in alkaline solution was relatively slow in the absence of laccase, resulting in weak CL emission. Second, the reactions of luminol−H_2O_2 and luminol−O_2 were catalyzed and accelerated by the addition of laccase. Consequently, more luminols in the ground state were oxidized into excited-state 3-aminophthalate anions. Accordingly, CL intensity increased when more 3-aminophthalate ions jumped from the excited to the ground state.

Analytical Characteristics for *E. coli* O157:H7 Detection. GOx is a low-cost, stable redox enzyme that can catalyze glucose to produce H_2O_2. On the basis of this principle, GOx can be used for glucose detection. GOx-based biosenors have recently attracted increased research interest. GOx is often used in conjunction with horseradish peroxidase (HRP) for bioenzyme-based signal amplification.[37,38] In addition, GOx has also been used as a label in immunosensors.[39,40] However, the application of GOx-laccase bienzyme-based immunosensors has not been reported.

Two steps are involved in this newly developed method for *E. coli* O157:H7 detection. First is the formation of IMBs based on the biotin−avidin and antibody−antigen (*E. coli* O157:H7) recognition reactions. The IMBs were then exposed to a solution containing glucose, and GOx potentially catalyzed the conversion of glucose into gluconic acid and H_2O_2. Second, the supernatant containing H_2O_2 was mixed with laccase and luminol, and H_2O_2 concentration was determined based on the CL reaction of luminol−H_2O_2−lacasse. Figure 2A shows the representative CL responses in a series of *E. coli* O157:H7 concentrations under optimal experimental conditions (optimization studies, Figures S1 and S2 in the Supporting Information). The CL signal linearly increased with increased *E. coli* O157:H7 concentration from 4.3×10^3 to 4.3×10^5 CFU mL^{-1}, and the total assay time was <2.0 h without any enrichment. The regression equation was $Y = 1.0752 \times X - 0.4976$, where Y is the base-10 logarithm of the CL intensity, and X is the base-10 logarithm of the concentration of *E. coli* O157:H7. The coefficient of determination (r^2) was 0.995. The LOD of 1.2×10^3 CFU mL^{-1} was calculated from the IUPAC definition[41] (3σ), which was considerably lower than that of the ELISA method (1.0×10^5 CFU mL^{-1}). This result suggested that the developed CL method had higher sensitivity and was superior to the previously reported HRP-based CL methods for the *E. coli* O157:H7 measurement.[14−16] The laccase-based strategy had high catalytic activity in strong alkaline medium compared with the HRP-based CL strategy, which was compatible with the luminol system. Furthermore, this developed method was ecofriendly and did not use any other chemical enhancer, such as 4-iodophenol, bromophenol blue,[42] 4-(1,2,4-triazol-1-yl)phenol,[43] and aryl boronic acid derivatives.[44,45] The LOD for PCR/electrophoresis was found to be 4.0×10^4 CFU mL^{-1} (Figure 2B, lane 5 in gel electrophoresis). The sensitivity of this developed method for detecting *E. coli* O157:H7 was about 1 order of magnitude higher than that of the conventional PCR/electrophoresis method. The reproducibility of the laccase-based MBs-CLEIA measurements was evaluated from 1.7×10^4 CFU mL^{-1} *E. coli* O157:H7 variability data of eleven replicates (n = 11) for which the relative standard deviation (RSD) was 3.5%. This result indicated that the proposed immunosensor had sufficient reproducibility for the detection of *E. coli* O157:H7.

Figure 2C shows that 1×10^5 CFU mL^{-1} *E. coli* O157:H7 was spiked with 1×10^5 CFU mL^{-1} *Salmonella*, *E. coli* DH5α, *ExPEC* 97, *ExPEC* 80, and *Streptococcus suis*. The CL signals obtained for *Salmonella*, *E. coli* DH5α, *ExPEC* 97, *ExPEC* 80, and *S. suis* were similar to the negative control signal, whereas the solution of the mixture of pathogens produced a signal similar to that of *E. coli* O157:H7. Figure 2D shows the corresponding electrophoresis images of the PCR products. Similarly, no electrophoresis band was observed for *Salmonella*, *E. coli* DH5α, *ExPEC* 97, *ExPEC* 80, *S. suis*, *E. coli* O91, *E. coli* O97, *E. coli* O149, and negative control (Figure 2D, lanes 3 to 7 and 13), whereas the mixture of both pathogens (Figure 2D, lanes 8 to 12) and *E. coli* O157:H7 (Figure 2D, lane 2) exhibited a unique positive electrophoresis band that produced only the expected 250 bp fragments. This phenomenon corresponded to the amplification of the IS200 insertion sequence that was specific for *E. coli* O157:H7. The result was the same as that obtained by this developed CL method. Selectivity was also demonstrated by challenging the sensor with bacteria more closely resembling *E. coli* O157:H7, such as other enterohemorrhagic *E. coli* O91, *E. coli* O97, and *E. coli* O149 (Figure 2E and 2F). These finding demonstrated the method's high specificity toward *E. coli* O157:H7.

Reliability was evaluated by recovery experiments in real samples using the standard addition method. *E. coli* O157:H7 concentration in the samples was then analyzed using the proposed method (Table 1) and found to range from 95.2% to 105.6% after finding that standard additions were satisfactory. These results demonstrated that the proposed sensor can be used in real water samples and had considerable practical applications. The proposed CL method was used to detect *E. coli* O157:H7 in synthesized samples (spring water, apple juice, and skim milk). *E. coli* O157:H7 concentrations were obtained using a conventional calibration curve and following a standard addition methodology. Table 2 shows that the results obtained from the developed CL method were consistent with those obtained from an alternative method (ELISA). Thus, the novel CL method had high potential for the analysis of *E. coli* O157:H7 in real samples.

Table 1. Recoveries for Several *E. coli* O157:H7−Spiked Real Samples

sample	added (CFU mL^{-1})	found (CFU mL^{-1})	RSD (%) (n = 5)	recovery (%)
spring water 1	8.30×10^3	8.00×10^3	1.95	96.4
spring water 1	1.24×10^4	1.18×10^3	2.34	95.2
spring water 3	1.24×10^5	1.31×10^5	2.62	105.6
apple juice 1	8.30×10^3	8.10×10^3	2.50	97.6
apple juice 2	1.24×10^4	1.22×10^4	4.99	98.4
apple juice 3	1.24×10^5	1.28×10^3	1.63	103.2
skim milk 1	1.24×10^4	1.22×10^4	2.70	98.4
skim milk 2	6.20×10^4	6.34×10^4	3.32	102.3
skim milk 3	1.24×10^5	1.29×10^5	3.32	104.0

CONCLUSIONS

A novel magnetic CL immunoassay for detecting of *E. coli* O157:H7 was developed. Its effectiveness was demonstrated

dx.doi.org/10.1021/ac4028774 | *Anal. Chem.* 2014, 86, 1115−1122

Table 2. Analytical Results of *E. coli* O157:H7 in Real Samples Obtained Using the Presented CL Method and by ELISA

sample	added (CFU mL^{-1})	found (CFU mL^{-1}) (n = 5)	ELISA (CFU mL^{-1}) (n = 5)
skim milk	1.50×10^6	$(1.47 \pm 0.06) \times 10^6$	$(1.60 \pm 0.05) \times 10^6$
apple juice	2.00×10^5	$(2.10 \pm 0.01) \times 10^5$	$(1.92 \pm 0.04) \times 10^5$
spring water	2.50×10^5	$(2.43 \pm 0.03) \times 10^5$	$(2.65 \pm 0.02) \times 10^5$

based on the enhanced CL of the luminol–H_2O_2 system catalyzed by laccase and on the conversion of glucose into H_2O_2 catalyzed by GOx. The luminophore of the luminol–H_2O_2–laccase system was still the excited-state 3-aminophthalate anion. The CL enhancement of laccase was suggested to be due to the catalysis of laccase on luminol oxidation by molecular O_2 as well as on the radical generation of $O_2^{\bullet-}$ and electron-transfer processes, during the luminol CL reaction. These characteristics showed the usefulness of laccase as an enhancer for the luminol–H_2O_2 CL system. Thus, laccase could be used for the sensitive determination of H_2O_2 and H_2O_2-based biological reactions. The synergy between the immunoassay and MBs resulted in enhanced sensitivity and selectivity of detection, as well as in the removal of interferences from other species. Furthermore, the advantages of the proposed method such as low cost, easy preparation, and time efficiency made this developed method suitable for the rapid and sensitive detection of *E. coli* O157:H7. The proposed protocol has potential applications in various fields, such as immunoassay and DNA hybridization.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: yongganghu@mail.hzau.edu.cn. Tel: 0086-27-87280670. Fax: 0086-27-87280670.

Author Contributions

§These authors contributed equally. The manuscript was written through the contributions of all authors. All authors have approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by grants of the National Natural Science Foundation of China (Grant 31030065), the Hi-Tech Research and Development Program of China (863 Program 2012AA101601, 2012AA101304), the National Natural Science Foundation of China (Grant 20005005), and the State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University (Grant AML-200905).

■ REFERENCES

(1) Mead, P. S.; Slutsker, L.; Griffin, P. M.; Tauxe, R. V. *Emerging Infect. Dis.* **1999**, *5* (6), 841–842.

(2) Ivniski, D.; Abdel-Hamid, I.; Atanasov, P.; Wilkins, E. *Biosens. Bioelectron.* **1999**, *14*, 599–624.

(3) Yu, L. S. L.; Uknalis, J.; Tu, S. *J. Immunol. Methods* **2001**, *256*, 11–18.

(4) Sen, K.; Sinclair, J. L.; Boczek, L.; Rice, E. W. *Environ. Sci. Technol.* **2011**, *45*, 2250–2256.

(5) Elizaquível, P.; Sánchez, G.; Aznar, R. *Food Control* **2012**, 25704–25708.

(6) Johnson, R. P.; Durham, R. J.; Johnson, S. T.; Macdonald, L. A.; Jeffery, S. R.; Butman, B. T. *Appl. Environ. Microbiol.* **1995**, 386–388.

(7) Liu, Y.; Li, Y. *Anal. Chem.* **2001**, *73*, 5180–5183.

(8) Akanda, M. R.; Tamilavan, V.; Park, S.; Jo, K.; Hyun, M. H.; Yang, H. *Anal. Chem.* **2013**, *85*, 1631–1636.

(9) Radke, S. M.; Alocilja, E. C. *Biosens. Bioelectron.* **2005**, *20*, 1662–1667.

(10) Jiang, X.; Wang, R.; Wang, Y.; Su, X.; Ying, Y.; Wang, J.; Li, Y. *Biosens. Bioelectron.* **2011**, *29*, 23–28.

(11) Gehring, A. G.; Albin, D. M.; Bhunia, A. K.; Reed, S. A.; Tu, S.; Uknalis, J. *Anal. Chem.* **2006**, *78*, 6601–6607.

(12) Huang, C.-J.; Dostalek, J.; Sessitsch, A.; Knoll, W. *Anal. Chem.* **2011**, *83*, 674–677.

(13) Subramanian, A.; Irudayaraj, J.; Ryan, T. *Biosens. Bioelectron.* **2006**, *21*, 998–1006.

(14) Magliulo, M.; Simoni, P.; Guardigli, M.; Michelini, E.; Luciani, M.; Lelli, R.; Roda, A. *J. Agric. Food Chem.* **2007**, *55*, 4933–4939.

(15) Donhauser, S. C.; Niessner, R.; Seidel, M. *Anal. Chem.* **2011**, *83*, 3153–3160.

(16) Wolter, A.; Niessner, R.; Seidel, M. *Anal. Chem.* **2008**, *80*, 5854–5863.

(17) Pappert, G.; Rieger, M.; Niessner, R.; Seidel, M. *Microchim. Acta* **2010**, *168*, 1–8.

(18) Gehring, A. G.; Irwin, P. L.; Reed, S. A.; Tu, S.; Andreotti, P. E.; Akhavan-Tafti, H.; Handley, R. S. *J. Immunol. Methods* **2004**, *293*, 97–106.

(19) Fan, A.; Lau, C.; Lu, J. *Anal. Chem.* **2005**, *77*, 3238–3242.

(20) Bi, S.; Yan, Y.; Yang, X.; Zhang, S. *Chem.—Eur. J.* **2009**, *15*, 4704–4709.

(21) Qin, G.; Zhao, S.; Huang, Y.; Jiang, J.; Ye, F. *Anal. Chem.* **2012**, *84*, 2708–2712.

(22) El-Boubbou, K.; Gruden, C.; Huang, X. *J. Am. Chem. Soc.* **2007**, *129*, 13392–13393.

(23) Chan, K. Y.; Ye, W. W.; Zhang, Y.; Xiao, L. D.; Leung, P. H.M.; Li, Y.; Yang, M. *Biosens. Bioelectron.* **2013**, *41*, 532–537.

(24) Zhao, L.; Sun, L.; Chu, X. *Trends Anal. Chem.* **2009**, *28* (4), 404–415.

(25) Wang, C.; Wu, J.; Zong, C.; Xu, J.; Ju, H.-X. *Chin. J. Anal. Chem.* **2012**, *40* (1), 3–10.

(26) Li, Y.-J.; Ma, M.-J.; Zhu, J.-J. *Anal. Chem.* **2012**, *84*, 10492–10499.

(27) Tang, J.; Tang, D.; Li, Q.; Su, B.; Qiu, B.; Chen, G. *Anal. Chim. Acta* **2011**, *697*, 16–22.

(28) Blanford, C. F.; Heath, R. S.; Armstrong, F. A. *Chem. Commun.* **2007**, 1710–1712.

(29) Wei, B.; Li, F.; Yang, H. C.; Yu, L.; Zhao, K. H.; Zhou, R.; Hu, Y. G. *Biosens. Bioelectron.* **2012**, *35*, 390–393.

(30) Alexandre, G.; Zhulin, I. B. *Trends Biotechnol.* **2000**, *18*, 41–42.

(31) Champagne, P. P.; Ramsay, J. A. *Appl. Microbiol. Biotechnol.* **2005**, *69*, 276–285.

(32) Couto, S. R.; Herrera, J. L. T. *Biotechnol. Adv.* **2006**, *24* (5), 500–513.

(33) Kiiskinen, L. L.; Viikari, L.; Kruus, K. *Appl. Microbiol. Biotechnol.* **2002**, *59* (2–3), 198–204.

(34) Merényi, G.; Lind, J.; Eriksen, T. E. *J. Biolumin. Chemilumin.* **1990**, *5*, 53–56.

(35) Kricka, L. J.; Voyta, J. C.; Bronstein, I. *Methods Enzymol.* **2000**, *305*, 370–390.

(36) Burdo, T. G.; Seitz, W. R. *Anal. Chem.* **1975**, *47*, 1639–1643.

(37) Le Goff, G. C.; Blum, L. J.; Marquette, C. A. *Anal. Chem.* **2011**, *83*, 3610–3615.

(38) Zeravik, J.; Ruzgas, T.; Fránek, M. *Biosens. Bioelectron.* **2003**, *18*, 1321–1327.

(39) Singh, A.; Park, S.; Yang, H. *Anal. Chem.* **2013**, *85* (10), 4863−4868.
(40) Lai, G.; Yan, F.; Ju, H. *Anal. Chem.* **2009**, *81*, 9730−9736.
(41) IUPAC, *Compendium of Chemical Terminology (the 'Gold Book')*, 2nd ed.; McNaught, A. D., Wilkinson, A., Eds.; Blackwell Scientific Publications: Oxford, 1997.
(42) Bi, S.; Yan, Y.; Yang, X.; Zhang, S. *Chem.—Eur. J.* **2009**, *15*, 4704−4709.
(43) Yang, X.; Guo, Y.; Mei, Z. *Anal. Biochem.* **2009**, *393*, 56−61.
(44) Kricka, L. J.; Cooper, M.; Ji, X. Y. *Anal. Biochem.* **1996**, *240*, 119−125.
(45) Kuroda, N.; Kawazoe, K.; Nakano, H.; Wada, M.; Nakashima, K. *Luminescence* **1999**, *14*, 361−364.