Chemiluminescent Probes

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Dextran-Based Polymeric Chemiluminescent Compounds for the Sensitive Optical Imaging of a Cytochrome P450 Protein on a Solid-Phase Membrane**

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Chemiluminescence (CL) has been exploited within a wide range of applications in many scientific fields.[1] CL imaging represents a promising detection system that is increasingly used for the ultrasensitive quantification and localization of several analytes. Currently, microarray technology has gained in popularity for the analysis of biological samples because of its benefits in the simultaneous detection of multiple analytes.[2] A CL signal is generally measured by a charge-coupled device (CCD) camera and then quantified by imaging software in a computer. For CL imaging, the traditional method usually employs horseradish peroxidase (HRP) or alkaline phosphatase as a signal enzyme, although the reduced stability of the enzyme at room temperature and high background interference limit the applicability of the technique in clinical analyses, especially for serum samples.[3] Thus, the development of a nonenzymatic CL-imaging probe is encouraged.

Herein, we report a simple method for synthesizing dextran-based chemiluminescent compounds and their application as CL-labeling macromolecular probes for the sensitive CL imaging of a cytochrome P450 (CYP) protein on a poly(vinylidene difluoride) (PVDF) membrane (Figure 1). The dextran-based chemiluminescent compound was tethered with a small amount of biotin as a linker and a large amount of luminol or isoluminol as CL emitter. Luminol and isoluminol are known for their CL properties, and their mechanistic details have been described.[4] In addition, the avidin–biotin interaction has been recognized in immunohistochemistry, enzyme-linked immunosorbent assay, and molecular biology.[5] The affinity of biotin binding to avidin is extremely high with an association constant of $10^{15}\,M^{-1}$.

To obtain a good signal strength and high sensitivity, extensive work on the synthesis of luminol and isoluminol derivatives is desirable to search for a novel nonenzymatic probe. We first synthesized luminol- or isoluminol-containing

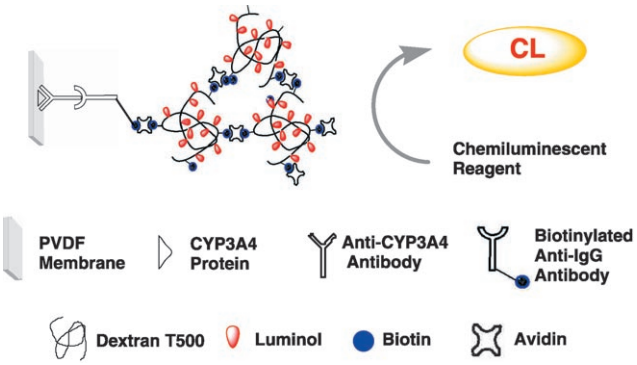
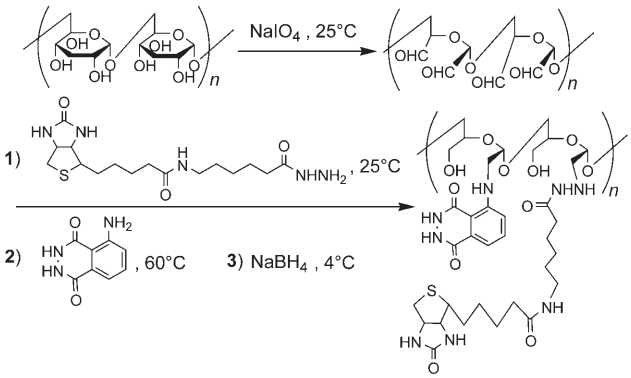


Figure 1. Detection of CYP3A4 (cytochrome P450, family 3, subfamily A, polypeptide 4) on a PVDF membrane with a polymeric dextran-based chemiluminescent probe. IgG = immunoglobulin G.

dextran T500 (average molecular weight 5×10^5 Da) chemiluminescent probes, which were tethered with biotin according to the procedure represented in Scheme 1. Biotin is a key



Scheme 1. Synthesis of dextran-based chemiluminescent compounds; see Experimental Section for details.

molecule that facilitates the extension of the structural framework of the chemiluminescent dextran. According to data from elemental analysis, the atomic composition of one of the synthesized dextran-based chemiluminescent probes was: C 43.0, H 5.8, N 4.4, and S 0.17%, and its molecular weight was approximately 6.3×10^5 Da. The data show that the probe contains 560 luminol units and 34 biotin units in a dextran T500 molecule (3100 glucose units), termed (Lu)560-(biotin)34-(Glc)3100. The increased introduction of luminol or isoluminol gave the probe a higher CL intensity.

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Figure 2a and b show gel-filtration chromatograms of luminol and the dextran T500-based chemiluminescent probe, respectively. Free luminol eluted at a retention time of 25 min, whereas the dextran-based probe was identified at

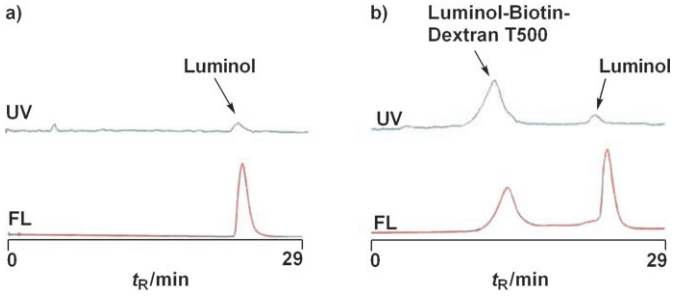

Figure 2. GFLC of dextran-based chemiluminescent probe: a) luminol (0.011 mg mL^{-1}); b) (Lu)560-(biotin)34-(Glc)3100 (1.0 mg mL^{-1}). GFLC conditions: injection volume, 10 mL; column, TSK gel T2000SW; eluent, 0.1% (v/v) aqueous solution of trifluoroacetic acid; flow rate, 1.0 mL min^{-1}; UV detection, λ_{abs} = 275 nm; fluorescence (FL) detection, excitation (mercury lamp)/emission (wavelength cutoff filter) = 254/ > 360 nm.

14 min. A small amount of free luminol (approximately 1%, w/w) was also observed in the probe as an impurity. However, the free luminol did not interfere with the detection of a target protein on a membrane, because the free luminol in the product could be sufficiently removed from the membrane by washing with a mixture of 0.15% Triton X-100 and phosphate-buffered saline (PBS; 10 mM), followed by an aqueous 50–100% methanol solution.

We previously reported a nonenzymatic procedure that employed a $CH_3CN/Na_2CO_3/H_2O_2$ system for CL with luminol in aqueous solution.[3] In a slight modification of the procedure, the employment of tetrapropylammonium hydroxide (TPA) instead of Na_2CO_3 gave a significant increase of CL intensity (> 20 times). In addition, Kyaw et al. reported that CL intensity could be enhanced by transition-metal catalysis.[6] Therefore, it was interesting to further improve our CL-emitting system by metal catalysis. Encouragingly, the highest CL intensity (> 8 times) from the chemiluminescent probe was observed when the CL-emitting reagents CH_3CN, TPA, and H_2O_2 were mixed with $FeCl_3$ (0.45 mM). The kinetics of this CL reaction was very fast and lasted approximately 80 s, with the most intensive signal 40 s after the start of the reaction. This short measurement time was advantageous for saving computer accumulation of enormous signals of CL imaging in the limited capacity of a hard disk. As little as 1.0 fmol of the dextran-based chemiluminescent probe could be sensitively visualized on a nylon membrane. The CL intensity was directly proportional to the concentration of the chemiluminescent probe ($y = 0.1362x + 0.0843$, $R^2 = 0.9917$).

From the Scatchard plot method, the binding constant K_a of the dextran-based probe to avidin on a membrane was 5.1×10^6. The formation of the extending framework for probe-chain assembly depended on the linkage of the biotin moieties of the chemiluminescent probe to four binding sites of avidin. Thus, the conditions for this process were optimized by investigating several concentrations of both avidin and the chemiluminescent probe (Figure 3). The optimal ratio of the dextran-based probe and avidin was 1:1 by weight. At this ratio, the polymeric chemiluminescent probe gave the highest CL intensity for the detection of the target CYP3A4 protein.

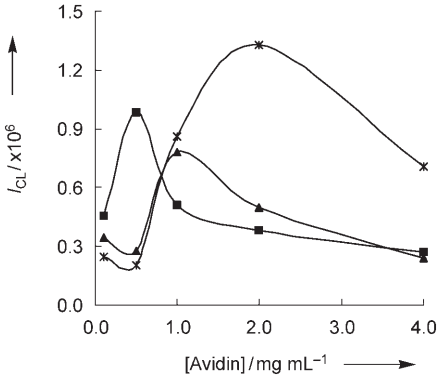

Figure 3. Effect of the concentration of avidin on the formation of an extending polymeric framework of the dextran-based chemiluminescent probe. CYP3A4 protein (750 fmol per spot) was employed on a PVDF membrane. The (Lu)560-(biotin)34-(Glc)3100 probe at 0.5 (■), 1 (▲), or 2 mg mL^{-1} (×) was mixed with avidin (0.1–4 mg mL^{-1}). The detection protocol was almost the same as that used in Figure 4, except that the amounts of avidin and probe were varied.

It is known that the sensitivity of the immunoassay could be greatly improved by attaching a number of chemiluminescent or fluorescent compounds of low molecular weight to a secondary antibody.[7] Thus, we employed this luminol- and biotin-containing dextran-based chemiluminescent macromolecular probe for the sensitive optical imaging of a specific protein on a PVDF membrane by the formation of a probe-chain assembly based on the interaction between avidin and biotin. We set up an immunoassay to detect CYP3A4 protein on a PVDF membrane. This membrane facilitated a higher absorption of proteins than the nylon membrane, and its hydrophobic property minimized the nonspecific interaction between the membrane and the dextran probe.

As shown in Figure 4a and b, at least 190 fmol of CYP3A4 on the PVDF membrane could be selectively detected by our system. The CL intensity was directly proportional to the concentration of CYP3A4 (in femtomoles per spot) on the membrane ($y = 949401x + 33975$, $R^2 = 0.9972$). Figure 4c and d show CL imaging data using an enzyme (HRP)-labeled avidin probe. The assay conditions were similar to those of the present system. The dextran-based probe gave lower background CL signals than the protein-based HRP probe. It is suggested that the dextran-based probe is more hydrophilic than the protein-based probe, and thus not readily absorbed on the PVDF membrane.

In conclusion, dextran-based chemiluminescent compounds containing luminol (or isoluminol) and biotin were successfully synthesized. At least 1 fmol of the chemiluminescent probe on a nylon membrane could be detected by use of the reagents CH_3CN, TPA, and H_2O_2 catalyzed by Fe^{III}. The extending polymeric framework of the dextran-based probe was simply formed by mixing avidin and the probe in a ratio of

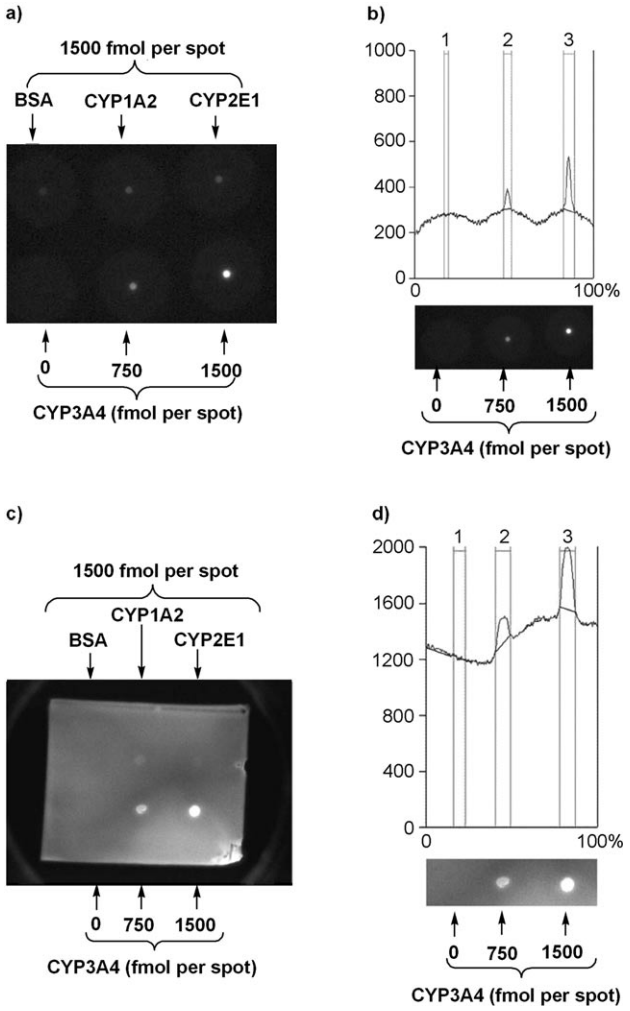

Figure 4. CL images of CYP3A4 on a PVDF membrane detected by a, b) a polymeric dextran-based probe and c, d) an enzyme-labeled avidin probe (see Experimental Section for details). BSA = bovine serum albumin.

1:1 (w/w). The probe-chain assembly produced enhanced the CL intensity, and thus sensitively and selectively detected CYP3A4 at concentrations as low as 190 fmol on a PVDF membrane after binding two kinds of antibody: a specific antibody for CYP3A4 and a biotinylated antibody for IgG. Therefore, this newly developed dextran-based chemiluminescent probe provides one of the most rapid and sensitive detection methods for CL imaging of proteins, and is complementary to the currently available enzymatic CL imaging. Ongoing research aims to extend our developed system to the detection of various proteins on a membrane microchip.

Experimental Section

Synthesis of chemiluminescent compounds: Dextran T500 (400 mg) was dissolved in water (60 mL) and the solution was mixed with sodium periodate (317 mg).[8] After approximately 30% oxidation, the partially oxidized dextran was precipitated with methanol and subsequently dissolved in dimethyl sulfoxide (60 mL). 6-Hydrazidohexyl D-biotinamide (30 mg) was added and the mixture was stirred at room temperature for 3 h. Luminol or isoluminol (80–240 mg) and glacial acetic acid (16 mL) were added and the mixture was stirred overnight at 60 °C. The modified dextran was precipitated with methanol and dissolved in ethylene glycol (30 mL). Sodium borohydride (870 mg) was added and the mixture was stirred at 4 °C for 4 h. The resultant dextran (approximately 280 mg), which contained luminol (or isoluminol) and biotin, was precipitated with methanol and dried in vacuo. Its purity was checked by gel-filtration liquid chromatography (GFLC). Detection of CYP3A4 with polymeric dextran-based probe: A PVDF membrane was spotted with ethanol followed by BSA and several human recombinant CYP proteins in aqueous solution (2 μL each). After drying, the membrane was incubated at 37 °C for 1 h with anti-human CYP3A4 rabbit polyclonal antibody (5.7 $\mu g\,mL^{-1}$) and biotinylated anti-rabbit IgG goat antibody (16.0 $\mu g\,mL^{-1}$) in a probe-chain assembly mixture (2 mL) composed of (Lu)560-(biotin)34-(Glc)3100 probe (4 mg), avidin (4 mg), BSA (6 mg), dextran (6 mg), and PBS (10 mM). The probe assembly mixture was preincubated at 37 °C for 1 h. After the reaction, the membrane was washed with a mixture of 0.15% Triton X-100 and 10 mM PBS solution (15 mL × 3) followed by 75% methanol (2 mL). The membrane was dried at 37 °C for 10 min in vacuo, then immersed in a CL-emitting solution (700 μL CH_3CN and 300 μL 1.0 M TPA) followed by addition of 30% H_2O_2 (50 μL) and 10 mM $FeCl_3$ (50 μL). The membrane was allowed to stand at room temperature for 3 s before CL detection for 2.0 min with a CCD camera.

Detection of CYP3A4 with enzyme-labeled avidin probe: The spotting of proteins on the membrane was the same as in the experiment with the dextran-based probe. After drying, the membrane was blocked with 5% skimmed milk at 37 °C for 1 h, and then incubated at 37 °C for 1 h with anti-human CYP3A4 rabbit polyclonal antibody (5.7 $\mu g\,mL^{-1}$), biotinylated anti-rabbit IgG goat antibody (16.0 $\mu g\,mL^{-1}$), and avidin–HRP (0.05 $\mu g\,mL^{-1}$). After the reaction, the membrane was washed with a mixture of 0.15% Triton X-100 and 10 mM PBS (15 mL × 3), and then treated with an enzymatic CL detection kit consisting of H_2O_2, 4-iodophenol, and luminol, which is available as the LumiGLO system before CL detection for 2.0 min with a CCD camera.

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