RESULTS AND DISCUSSION

Elemental Analysis and Chromatography of Dextran-Based Polymeric CL Compounds. Four dextran-based polymeric CL compounds were prepared according to the procedure represented in Figure 2. Elemental analysis data of our synthesized dextran-based polymeric CL compounds confirmed their chemical compositions. According to the elemental analysis data, molecular weight and composition of these CL compounds were able to be deduced as shown in Table 1. The number of luminol or isoluminol units per a dextran molecule was increased as the molecular weight of dextran carrier increased. It is well known that the sensitivity of the immunoassay could be greatly improved by attaching with a number of signal labels into the secondary antibody.[24, 25] Thus we intended to employ these dextran-based polymeric CL compounds as sensitive signal-labeling probes for the optical imaging of a specific protein on a PVDF membrane. Most importantly, the increased labeling level did not deteriorate the biochemical activity of the labeled bio-molecules such as biotinylated antibodies, due to that the dextran molecule was herein used as carrier to bind many CL emitters and linker compounds together.

Figure 3 shows a representative gel-filtration chromatogram of luminol (a) and a dextranT500-based luminol and biotin-containing polymeric compound (b). All synthesized polymeric CL compounds (Luminol- or Isoluminol-Biotin-DextranT2000 or T500) were eluted at the same retention time. Free luminol or isoluminol was eluted at the retention time of 25 min, while the expected polymeric compounds were identified at 14 min. Although four dextran-based polymeric CL compounds were successfully synthesized, a small amount of free luminol or isoluminol was also contributed as an impurity (Figure 3b). In order to remove free luminol or isoluminol, we attempted different purification methods for synthesis and detection on membrane. It was found that, after binding these dextran-based polymeric CL compounds into nylon or PVDF membrane, only washing the membrane with 50-100 % methanol was sufficient to remove free luminol or isoluminol from these polymeric CL compounds.

Table 1. Elemental Analysis of Luminol or Isoluminol-Biotin-Dextran Compounds

	Original Data				Calculated Composition	
	C%	H%	N%	S%	Composition of Compound	MW
Luminol-Biotin-Dextran T2000	44.355	5.845	5.53	0.455	$(Lu)_{2627}$-$(Biotin)_{385}$-$(Glc)_{12346}$	2701209
Luminol-Biotin-Dextran T500	43.62	5.845	4.385	0.17	$(Lu)_{559}$-$(Biotin)_{34}$-$(Glc)_{3086}$	631556
Isoluminol-Biotin-Dextran T2000	43.685	5.65	5.955	0.31	$(IL)_{3262}$-$(Biotin)_{275}$-$(Glc)_{12346}$	2837018
Isoluminol-Biotin-Dextran T500	42.02	5.845	2.775	0.08	$(IL)_{321}$-$(Biotin)_{15}$-$(Glc)_{3086}$	593056

Lu=luminol unit; IL=isoluminol unit; Glc= glucose unit.

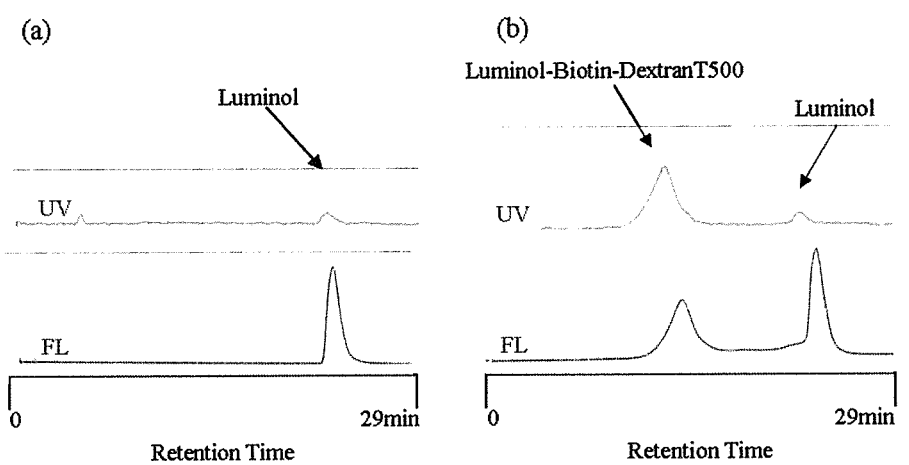
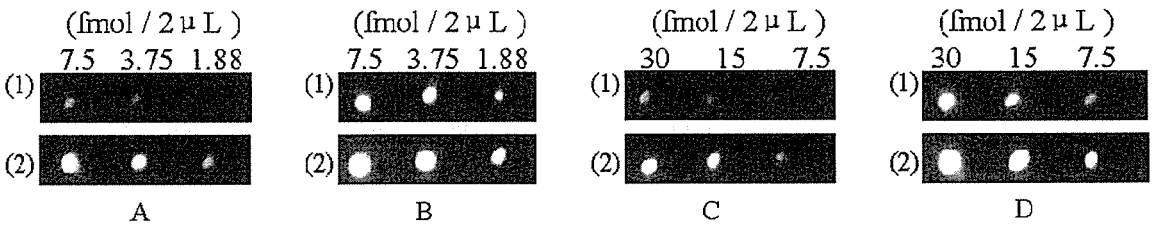


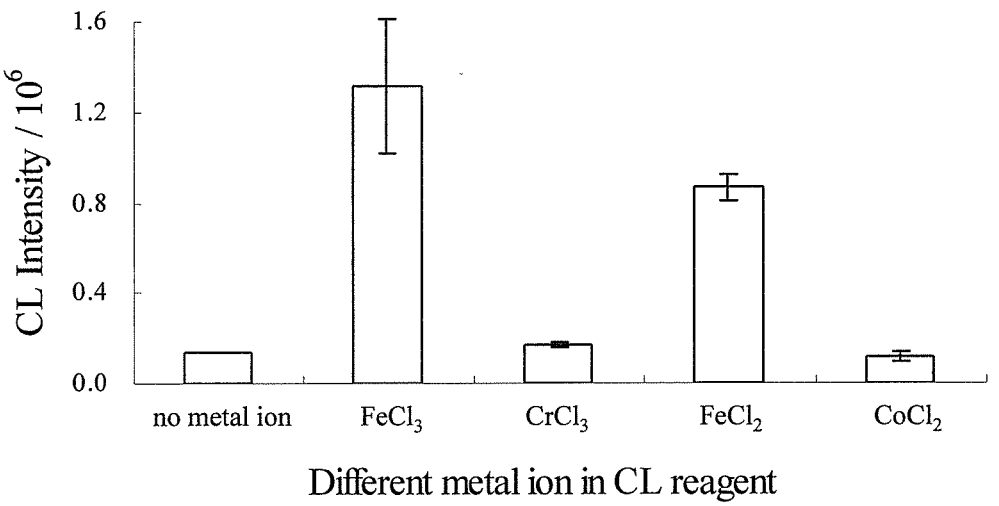
Figure 3. GFLC of Luminol-Biotin-DextranT500 polymeric CL compound. Samples: (a) 0.011 mg / mL luminol at 25 min; (b) 1.0 mg/mL Luminol-Biotin-DextranT500 $[(Lu)_{559}$-$(Biotin)_{34}$-$(Glc)_{3086}]$ at 14 min. GFLC conditions: injection volume, 10 μL; column, TSK gel T2000SW; eluent, 0.1 % (v/v) TFA aqueous solution; flow rate, 1.0 mL/min; UV detection, λabs=275 nm; FL detection, Ex (mercury lamp)/Em (wavelength cut-filter) =254 nm / > 360 nm.

CL Imaging of Dextran-Based Polymeric CL Compounds on Nylon Membrane. We previously reported a non-enzymatic CL procedure employing CH_3CN-Na_2CO_3-H_2O_2 system for CL imaging.[18,19] As the synthesized polymeric compounds were easily dissolved in water and readily absorbed to relatively high polar (nylon) membrane, a slight modification of the corresponding procedure employing organic alkaline reagents (TBA or TPA) was tested. It was found that the employment of these TBA and TPA reagents gave a significant improvement of CL intensity (Figure 4a). In addition, Kyaw et al. reported that CL intensity could be enhanced by transition metal catalysis,[11] and CL intensity strongly depended on the metal-complex species as well as the concentration of metal ions.[10-14] Therefore it would be interesting to further improve our CL system by employing metal catalysis. Four metal ions, Fe (III), Fe (II), Co (II) and Cr (III) were screened for the improvement of CL intensity. Encouragingly, the highest CL intensity from the polymeric compound was observed when a CL reagent (CH_3CN-TPA-H_2O_2 system) was catalyzed by Fe (III) (Figure 4b). Although, the concentration at 0.94 mM of Fe (III) gave the highest intensity, however, precipitation of the Fe (III) complex was also observed. In order to overcome this problem, a concentration of Fe (III) at 0.45 mM was selected since at this concentration no precipitation of the Fe (III) complex was observed (Figure 4c). Promisingly, it was shown that as low as 1.0 fmol of Luminol-Biotin-DextranT500 could be sensitively visualized on the nylon membrane. CL intensity was shown to be directly proportional to the concentration of Luminol-Biotin-DextranT500 (fmol/2 μL) ($y = 0.1362x + 0.0843$, $R^2 = 0.9917$; where y is CL intensity, and x is the concentration of Luminol-Biotin-DextranT500).

(a)


(fmol / 2 μ L)
7.5 3.75 1.88
(1)
(2)
A
(fmol / 2 μ L)
7.5 3.75 1.88
(1)
(2)
B
(fmol / 2 μ L)
30 15 7.5
(1)
(2)
C
(fmol / 2 μ L)
30 15 7.5
(1)
(2)
D

(b)


CL Intensity / 10^6
1.6
1.2
0.8
0.4
0.0
no metal ion
FeCl3
CrCl3
FeCl2
CoCl2
Different metal ion in CL reagent

(c)

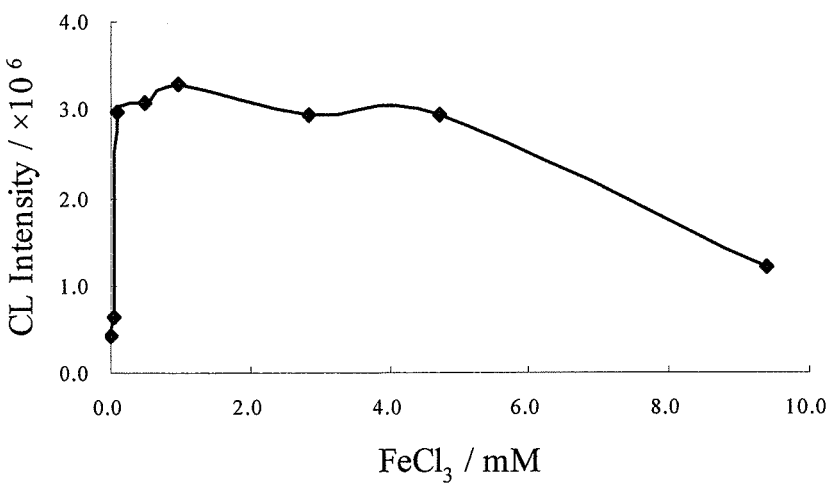

CL Intensity / ×10 6
4.0
3.0
2.0
1.0
0.0
0.0
2.0
4.0
6.0
8.0
10.0
FeCl3 / mM

Figure 4. (a) CL imaging of Luminol- or Isoluminol-Biotin-DextranT2000 or T500 compounds using two kinds of CL reagents. Polymeric compounds: (A), $(IL)_{3262}$-$(Biotin)_{275}$-$(Glc)_{12346}$; (B), $(Lu)_{2627}$-$(Biotin)_{385}$-$(Glc)_{12346}$; (C), $(IL)_{321}$-$(Biotin)_{15}$-$(Glc)_{3086}$; (D), $(Lu)_{559}$-$(Biotin)_{34}$-$(Glc)_{3086}$. CL reagents: (1), CH_3CN 700 μL, 0.5 M TBA 300 μL and 30% H_2O_2 50 μL; (2), CH_3CN 700 μL, 1.0 M TPA 300 μL, 30% H_2O_2 50 μL, and 10 mM $FeCl_3$ 50 μL. (b) Effect of several metal ions on CL intensity. $(Lu)_{559}$-$(Biotin)_{34}$-$(Glc)_{3086}$ (10 fmol/2 μL) was used for detection. (c) Effect of Fe (III) concentration on CL intensity. $(Lu)_{559}$-$(Biotin)_{34}$-$(Glc)_{3086}$ (20 fmol/2 μL) was used for detection.

Moreover, in the absence of Fe (III), these polymeric CL compounds have a slow CL kinetics and their CL signals lasted for 5 min on the nylon membrane. In the presence of Fe (III) ion, however, their CL kinetics was increased as shown in Figure 5, and CL intensity was significantly enhanced compared with a non-metal catalysis.

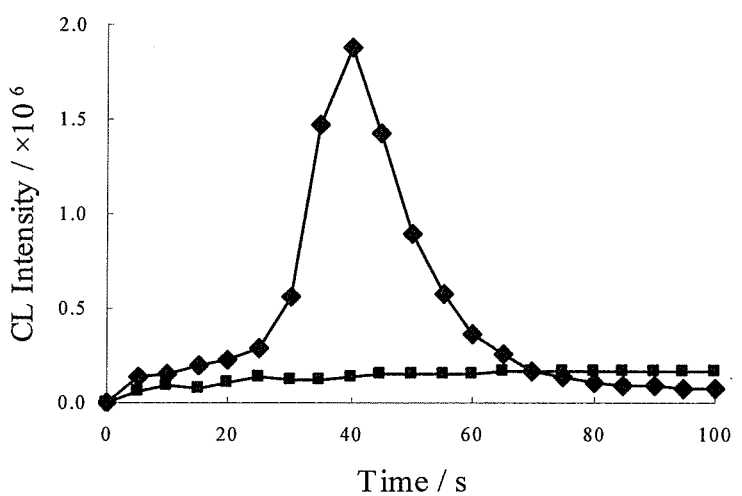


Figure 5. CL kinetics of Luminol–Biotin–Dextran T500 compound on nylon membrane. (■) without Fe (III) catalyst, (♦) with Fe (III) catalyst.

The Binding Ability of Polymeric Luminol- or Isoluminol-Biotin-Dextran Compounds to Avidin. The binding constant between the synthesized dextran-based polymeric CL compound and avidin on PVDF membrane was shown to be increased with the increased number of biotins (Table 2). However, this was not the case for $(IL)_{3262}$-$(Biotin)_{275}$-$(Glc)_{12346}$, where the binding constant of this CL compound was found to be minimal as compared with $(Lu)_{559}$-$(Biotin)_{34}$-$(Glc)_{3086}$. It was speculated that an excess amount of isoluminol in $(IL)_{3262}$-$(Biotin)_{275}$-$(Glc)_{12346}$ might attenuate its binding with avidin.

Table 2. Binding Ability of Different Dextran-Based Polymeric CL Compounds to Avidin

Compound	Binding Constant [K, M^{-1}]	Ratio of molar binding Avidin / Compound
$(Lu)_{2627}$-$(Biotin)_{385}$-$(Glc)_{12346}$	4.8×10^{7}	571
$(Lu)_{559}$-$(Biotin)_{34}$-$(Glc)_{3086}$	5.1×10^{6}	106
$(IL)_{3262}$-$(Biotin)_{275}$-$(Glc)_{12346}$	1.5×10^{6}	1014
$(IL)_{321}$-$(Biotin)_{15}$-$(Glc)_{3086}$	1.5×10^{5}	179

The formation of the extend-framework polymeric Luminol-Biotin-Dextran probe was depended on the linkage of biotin moieties of the polymeric compound to four binding sites of avidin. Thus, the formation condition was optimized by investigating various concentrations of avidin and Luminol-Biotin-DextranT500. The optimal ratio between the Luminol-Biotin-DextranT500 and avidin was found to be at 1:1 by weight (Figure 6). According to this ratio, the polymeric Luminol-Biotin-Dextran probe gave the highest CL intensity for the detection of the target protein CYP3A4.

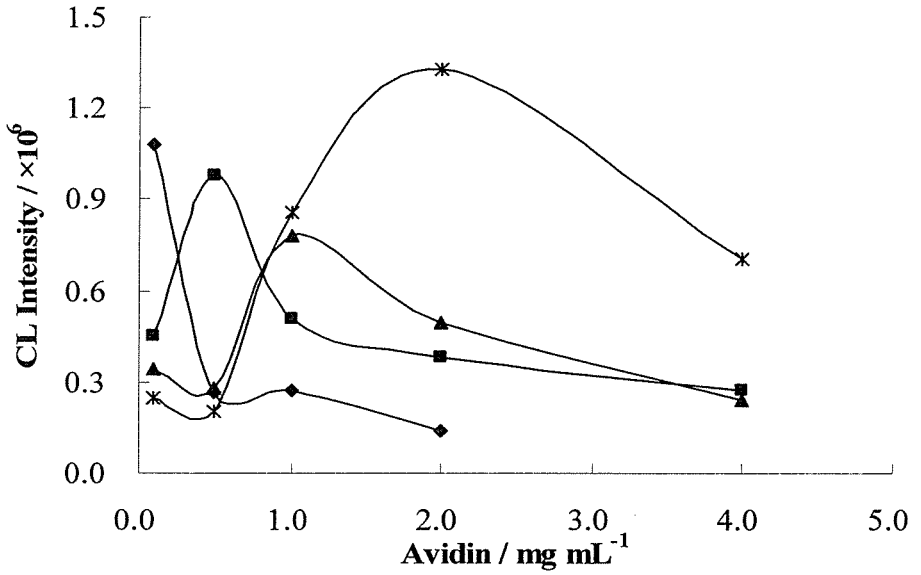


Figure 6. Effect of the concentration of avidin on the formation of extend-framework polymeric CL probe. CYP3A4 protein (760 fmol/2 μL) was employed on PVDF membrane. Luminol-Biotin-DextranT500 $[(Lu)_{559}\text{-}(Biotin)_{34}\text{-}(Glc)_{3086}]$ at (♦) 0.1 mg/mL, (■) 0.5 mg/mL, (▲) 1 mg/mL or (*) 2 mg/mL was mixed with 0.1-4 mg/mL avidin.

Detection of CYP3A4 Protein by Using Polymeric Luminol-Biotin-DextranT500 Probe. It was shown that our immunoassay was able to detect the CYP3A4 protein on the PVDF membrane. The PVDF membrane had a higher absorption to CYP3A4 than the nylon membrane. The hydrophobicity of PVDF membrane minimized the non-specific interaction between the membrane and the dextran probe. Therefore, PVDF membrane was chosen for this immunoassay. At least 190 fmol of CYP3A4 on the PVDF membrane was able to be detected by our system (Figure 7). It was shown that CL intensity had a directly proportional relationship to the concentration of CYP3A4 (fmol/2 μL) on the PVDF membrane ($y = 949401x + 33975$, $R^2 = 0.9972$, where y is CL intensity, and x is the concentration of CYP3A4). Overall, this newly promising method serves as one of alternative techniques in the immunoassay.

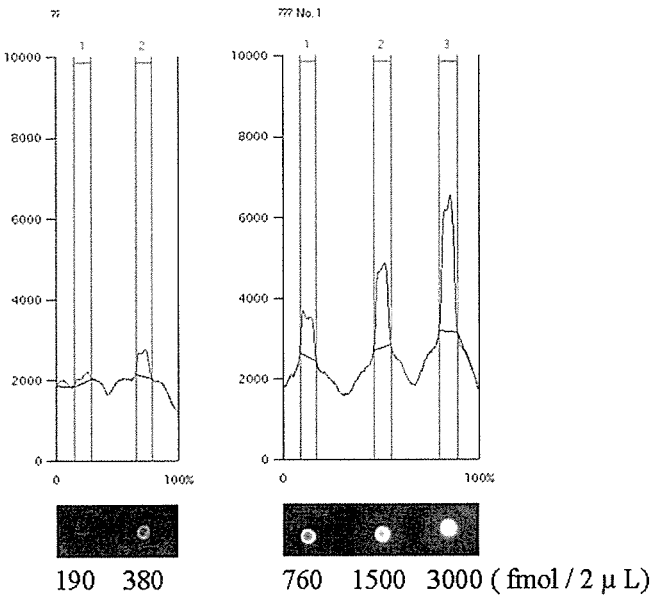


Figure 7. CL image of CYP3A4 on a PVDF membrane detected by polymeric Luminol-Biotin-Dextran probe.

CONCLUSIONS

Four Luminol- or Isoluminol-Biotin-Dextran CL compounds were successfully synthesized. The formation of the extend-framework polymeric dextran-based CL probe was able to be simply formed by mixing with avidin and the probe at 1:1(w/w) ratio. This formed polymeric dextran-based probe enhanced the CL intensity due to the increasing number of luminol or isoluminol units. At least 1 fmol of a Luminol-Biotin-Dextran CL compound on a nylon membrane was able to be detected with CH_3CN-TPA-H_2O_2 system catalyzed by Fe (III). Even as low as 190 fmol of CYP3A4 was able to sensitively detect with our newly developed probe. Therefore, this procedure serves as one of the useful and sensitive tools in biochemical analyses and is complementary to the currently available enzymatic CL imaging methods. Ongoing research is to extend our developed system for the detection of various CYP proteins.

ACKNOWLEDGMENTS

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Sensitive chemiluminescence-imaging detection of telomere DNA by use of luminol-modified macromolecular probe

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Keywords: Chemiluminescence detection; Telomere DNA; Dextran probe; Luminol; Non-enzymatic probe.

ABSTRACT

Chemiluminescence-imaging technology has been widely used in many fields of research, and a more facile and sensitive DNA-detection method has been desired for microanalysis. Herein we report a novel detection method of DNA utilizing a non-enzymatic chemiluminescent probe, in which many luminol and biotin molecules are covalently conjugated into one dextran molecule. This probe can be tethered with avidin to form a probe-chained assembly based on the interaction between free avidins and biotins in the probe. Avidin recognizes biotinylated cDNA that hybridizes with a target DNA, and also plays as "a hub" to link to the probe-chained assembly. Several chemiluminescent probes having luminol or isoluminol and biotin were synthesized using different sizes of dextran. The chemiluminescence intensity of the probes was increased with increased number of luminol incorporated into the dextran. DextranT2000 (average MW, 2,000 kDa) containing 2627 molecules of luminol and 385 molecules of biotin was used for the detection of telomere DNA. This probe afforded the sensitive detection of the target DNA at sub-picomole level, while problematic background signals were observed in a comparative study using a widely used horseradish peroxidase. The current method permitted a promising chemiluminescence-imaging detection of the target DNA.

INTRODUCTION

Various gene sequences as well as their functions have been discovered through the determination of entire DNA sequence in human genome. The technique to analyze a particular DNA sequence has been also in widespread use for various purposes such as detecting genetic diseases, finding gene function, DNA forensics and genetic markers. Hence easy, quick and highly sensitive detection of DNA is essential to achieve the high-throughput microanalysis for the determination of smaller amount of DNA. In recent decades, several fluorescent nucleic acid

probes have been developed instead of radioisotopes for DNA micro-array technology. For instance, molecular beacon utilizes the self-switching of "bright" and "dark" state by a fluorophore and a fluorescence quencher in one oligonucleotide probe (1-6). The phenomenon, of which the close proximity between a fluorophore and gold surface results in a quenching of fluorescence, is well combined with a strong affinity of a thiol group to a gold atom (7-11). The interaction between two fluorophores, such as fluorescence- resonance energy transfers (FRET) (12, 13) and monomer-excimer fluorescence change (14-16), is also applied for the DNA detection analysis. However, there exist known limits in the DNA analysis with a fluorescence-labeled probe. Those methods require the direct incorporation of fluorophores into cDNA probes, and their complex-formed hybridization causes a difficulty in designing probes.

On the other hand, a chemiluminescence (CL) detection technique has also become an important tool not only for nucleic acids hybridization assay but also for many fields of researches due to its high sensitivity, accurate quantification and simple instruction. Especially, a series of luminol compounds such as luminol and isoluminol is a long-standing chemiluminescent agent that emits light upon the reaction with hydrogen peroxide under basic conditions. It is also known that the luminescence of luminol compounds is enhanced by metal ions, phenols (17) and peroxidases such as horseradish peroxidase (HRP) (18). HRP catalyzes the CL reaction with hydrogen peroxide and luminol (or isoluminol), so that HRP has been used as a signal probe. A family of dioxetane compounds is known as a substrate for alkaline phosphatase, and produces a strong CL upon hydrolysis (19-25). These enzymatic luminescences sometimes reduce the reproducibility of detection signal caused by instability of the enzyme at ambient temperature (26), and the elongation of reaction time for the enzymatic luminescence significantly increases the background signal. Thus the development of a non-enzymatic chemiluminescent probe has been highly desired for microanalysis (27-38).

In this article, we demonstrate a sensitive method for the detection of telomere DNA using

newly synthesized dextran-based CL probes. This CL detection system requires avidin-viotin interaction, which has been widely utilized in biochemical analysis such as enzyme-linked immunosorbent assay (ELISA) (39-41). We designed the dextran-based CL probes that contain many biotin and luminol moieties for the conjugation with avidin and for the CL enhancement, respectively. The probes could aggregate each other via avidin molecules to form a probe-chained assembly (Figure 1). This detection system permitted a sensitive CL-imaging assay of teromere DNA on a nylon membrane. The comparative study on the same target DNA showed higher sensitivity and reproducibility for our method with a dextran-based CL probe than a HRP probe.

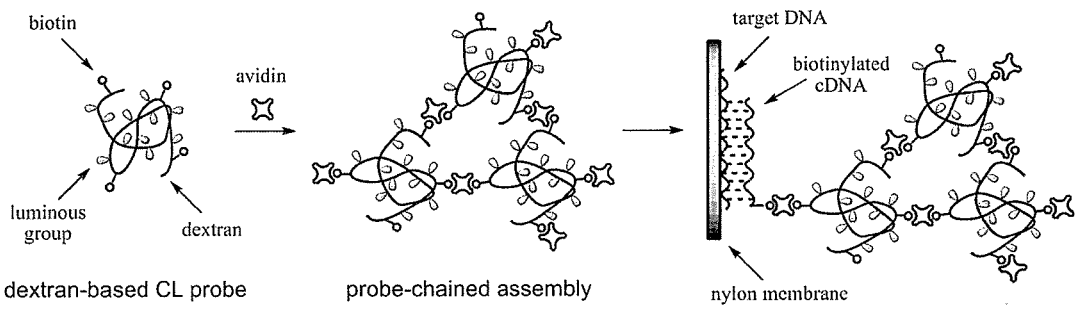


Figure 1. Schematic principle for sensitive CL detection of a target DNA by use of luminol- and biotin-containing dextran probe. Conjugation of the dextran-based probe via avidin protein resulted in the formation of a probe-chained assembly. This assembly recognized the biotinylated cDNA that hybridizes with the target DNA.

MATERIALS AND METHODS

Chemicals and reagents

DextranT2000 (average MW, 2000 kDa) and DextranT500 (average MW, 500 kDa) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Dextran800 (average MW, 800

kDa) and Dextran170 (average MW, 170 kDa) were obtained from Meito Sangyo (Nagoya, Japan). Avidin was purchased from Wako Pure Chemical Industries (Osaka, Japan). Luminol was purchased from Nacalai Tesque (Kyoto, Japan). Isoluminol was purchased from Tokyo Chemical Industry (Tokyo, Japan). 6-Hydrazidohexyl *D*-biotinamide was purchased from Dojindo Laboratory (Kumamoto, Japan). All DNAs used in this study were obtained from Sigma Genosys Japan (Hokkaido, Japan), and dissolved in 1.0 mM EDTA. Sequences of DNAs are as follows; biotinylated 18mer telomere cDNA [5'-biotin-$(CCCTAA)_3$], 60mer telomere DNA [5'- $(TTAGGG)_{10}$], 18mer telomere cDNA [5'- $(CCCTAA)_3$], 42mer telomere cDNA [5'- $(CCCTAA)_7$], 30mer nuclear factor κB single stranded DNA (NF- κB ssDNA) [5'-AGTTGAGGGGACTTTCCCAACTAGG AATCT], 30mer NF- κB cDNA [5'-AGATTCCTAGTTGGGAAAGTCCCCTCAACT]. Tetra-*n*-butylammonium hydroxide (TBA), bovine serum albumin (BSA), and HRP-avidin were obtained from Sigma (Missouri, USA). Triton X-100 was purchased from ICN Biomedicals (Ohio, USA). LumiGLO® Chemiluminescent Substrate Sysytem was purchased from KPL (Maryland, USA). A Nylon membrane (0.2 μm pore size) was purchased from ATTO (Tokyo, Japan).

General procedure for the synthesis of dextran probes

Oxidation of dextran. Dextran (400 mg, 2.5 mmol as glucose) was dissolved in 30-80 mL of water and precipitated by a slow addition into 400 mL of methanol with stirring. The precipitate was filtered, and the resulting purified dextran was re-dissolved in 50 mL of water. To this was added $NaIO_4$ (317 mg, 1.5 mmol) in 10 mL of water over 5 min with stirring at room temperature. The reaction was monitored by UV absorbance of $NaIO_4$ at 310 nm. At the point of UV spectrum being unchanged, the reaction mixture was poured into 300 mL of methanol with stirring. The mixture was allowed to stand without stirring until all precipitate was settled down. Methanol was removed by careful decantation and the remaining residue was re-dissolved in 45

mL of water. The suspension was warmed at 60°C to dissolve the white cloudiness completely, and the solution was cooled to room temperature. The solution was added to 400 mL of methanol dropwise with stirring and the mixture was allowed to stand until the precipitate settled down. The resulting mixture was then filtered off to give a pure oxidized dextran. The oxidized dextran was highly hygroscopic and kept in methanol as soon as filtration was finished.

Addition of biotin and luminol into oxidized dextran. The above wet oxidized dextran was added to 80 mL of DMSO and heated at 100°C with stirring until completely dissolving. After cooling to room temperature, 6-hydrazidohexyl *D*-biotinamide (30 mg, 80.7 μmol) was added to the dextran solution and the mixture was stirred at room temperature for 2 h and 40 min. Acetic acid (16 mL) followed by luminol (240 mg, 1.4 mmol) were added at room temperature and the mixture was left stirring at 60°C overnight. The reaction solution was added to 400 mL of methanol dropwise with stirring, and the resulting suspension was allowed to stand until the precipitate was settled down. Methanol was removed by careful decantation and another 500 mL of methanol was added to re-suspend the residue. After stirring for 30 min, the precipitate was collected by filtration to give a wet yellowish powder.

Reductive alkylation of imino-form of modified dextran. The product obtained above was added to 60 mL of ethylene glycol and heated at 60°C with stirring until completely dissolving. The mixture was then cooled in an ice bath, and $NaBH_4$ (3.48 g, 92.0 mmol) was added to it in four portions every hour over 3 h. The reaction was continued at 4°C overnight without stirring. The reaction solution was added dropwise to 300 mL of methanol with stirring, and the resulting suspension was allowed to stand until the precipitate was settled down. Methanol was removed by a careful decantation and 500 mL of methanol was added to re-suspend the residue. After stirring for 30 min, the precipitate was collected by filtration. The solid obtained was again

dissolved in 10 mL of water and re-precipitated with 100 mL of methanol followed by careful decantation, re-suspension with 100 mL of methanol with stirring for 30 min and subsequent filtration. The washing step was repeated twice. After filtration, the remaining residue on the filter paper was dried under reduced pressure to give a desired luminal- and biotin-containing dextran probe as a pale yellow powder (281 mg).

Chromatography and spectrophotometric measurement of the probes. Gel-filtration liquid chromatography (GFLC) was carried out by use of a UV-8011 UV detector (Tosoh, Japan), a Hitachi-Fluorescence Monitor (Hitachi, Japan), and a TSK G3000SW gel-filtration column (Tosoh, Japan). A portion (10 μl) of 1.0 mg/mL aqueous probe solution was applied to the column using 0.1 % (v/v) aqueous TFA as an eluent at the flow rate of 1.0 mL/min. The UV absorption was monitored at 275 nm and FL was monitored at excitation wavelength of 370 nm and emission wavelength of 430 nm. UV spectra were measured with a UV-240 (Shimazu, Japan) and the molar extinction coefficient of the probes was calculated at their maximum UV absorption wavelength. FL spectra were measured with a Spectrofluorometer FP-6300 (Jasco, Japan) and obtained as emission-excitation two dimensional spectra. For CL measurement, 50 pM probe in 0.1 M sodium carbonate buffer containing 10 % (v/v) acetonitrile were reacted with 20μL of 0.1 M aqueous H_2O_2 and the resulting CL was monitored for 10 min on a Luminescence Reader BLR-201 (Aloka, Japan).

DNA detection on a solid-phase membrane. Telomere DNA (0.2-1.6 pmol/2μL), 18mer and 42mer telomere cDNAs (1.6 pmol/2μL), 30mer NF- κB ssDNA (1.6 pmol/2μL) and 30mer NF- κB cDNA (1.6 pmol/2μL) were spotted on a nylon membrane, and the membrane was heated at 80°C for 10 min. The baked membrane was incubated with 300 pmol of biotinylated 18mer telomere cDNA (b-cDNA) dissolved in 600 μL of saline sodium citrate (SSC) buffer (150 mM

sodium chloride, 15 mM sodium citrate and 1 mM EDTA, pH 7.0) at 37°C for 2 h. The membrane was washed with 2 mL of SSC buffer for 5 min followed by incubation with 10 mg/mL BSA in 2 mL of SSC buffer and at 37°C for 2 h.

In case for the detection with dextran-based CL probe, the membrane was then treated with 1 mL of the probe-chained assembly solution containing 5 mg/mL BSA, different concentrations of luminol- and biotin-containing dextran probe (0.08-1.2 mg/mL) and avidin (0.04-1.4 mg/mL) at 37°C for 10 min. The membrane was then washed three times with 3 mL of 10 mM, pH 7.4 phosphate buffered saline (PBS) containing 1 mM EDTA and 5 % (v/v) of Triton X-100, followed by washing with 2 mL of 50 % aqueous methanol at 37°C for 5 min. After drying the membrane under reduced pressure for 10 min, the membrane was immersed in 1 mL of CL reaction solution consisted of 700 μl of acetonitrile, 270 μl of 0.5 M aqueous TBA and 30 μl of 30% aqueous H_2O_2 for 10 s and then CL imaging was detected with charge-coupled device (CCD) camera for up to 6 min by Light Capture AE-6962/C/FC supplied from ATTO (Tokyo, Japan).

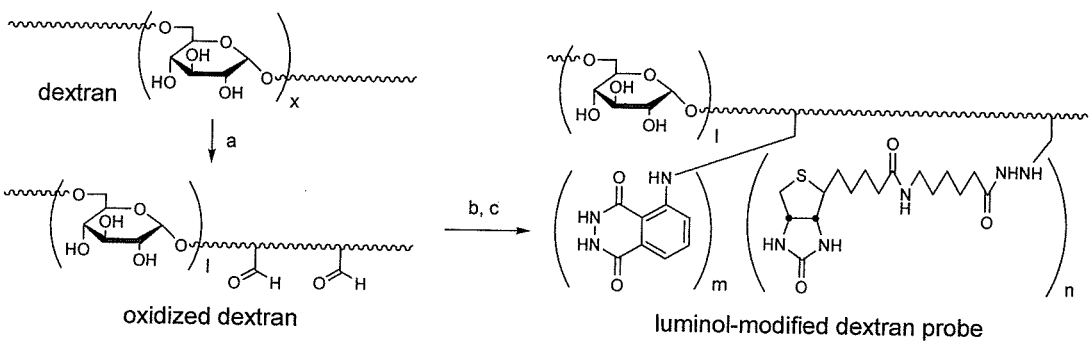
In case for the detection with HRP-avidin probe, the membrane after the hybridization with b-cDNA was treated with 1 mL of HRP-avidin probe in SSC buffer containing 5 mg/mL of BSA at 37°C for 10 min. The membrane was then washed three times with 3 mL of 10 mM PBS containing 1 mM EDTA and 5% (v/v) of Triton X-100. The membrane was then treated with a chemiluminescent detection kit consisted of hydrogen peroxide, 4-iodophonol and luminol available as LumiGLO® Chemiluminescent Substrate Sysytem. The resulting CL was detected with CCD camera for up to 6 min.

RESULTS AND DISCUSSION

Synthesis, structure determination, and chromatography of dextran-based probes

Dextran molecule is a good scaffold for the multivalent probe because a highly polar

characteristic of dextran retains the hydrophilicity even after the multiple modifications by hydrophobic moieties of luminal (Lu) or isoluminol (ILu). Also the treatment of dextran with sodium periodate can produce aldehyde moieties to which many functional groups can be introduced via reductive *N*-alkylation reaction (42). Thus, we obtained highly sensitive chemiluminescent (CL) probes having many Lu and biotin (Bi) molecules in one dextran molecule.



Scheme 1. Synthetic route to dextran-based CL probes. Reaction conditions: a, $NaIO_4$, water, room temperature; b, 6-hydrazidohexyl *D*-biotinamide in DMSO then acetic acid, luminol, 60°C; c, $NaBH_4$ in ethylene glycol, 4°C.

The synthetic route to the dextran-based CL probes is shown in Scheme 1. The synthesis was started with several different dextran molecules. In a preliminary study we found that an exceeded oxidation of dextran makes the product insoluble in any kind of solvents. Therefore 0.6 molar-equivalent of periodate was used to consume about 30 % of the glucose moieties in dextran to maintain its solubility in water. In order to find the best probe for the sensitive detection of DNA, we synthesized many probes having different amounts of Lu and Bi molecules. Thus, oxidized dextrans were treated with different amounts of 6-hydrazidohexyl *D*-biotinamide,

and consecutively reacted with different amounts of Lu or ILu for labelling. The product was isolated at this stage to remove unreacted Lu, and the resulting imino-form of the probe was then saturated by reductive amination using sodium borohydride. The desired crude product was purified carefully by dissolving in water and precipitating in methanol.

The structures of synthesized probes were determined by elemental analysis measuring the ratio of carbon, hydrogen, nitrogen and sulphur atoms. Since sulphur atom is included only in biotin moiety, the number of biotin conjugated in the probe can be calculated from the contents of sulphur atoms. This result also gives the number of nitrogen in biotin, therefore the number of Lu or Ilu attached can be obtained. Those results enable to count the number of carbon and hydrogen atoms in Bi and Lu or ILu, and the number of glucose moieties was finally calculated. The results clearly exhibit that the dextran probes having different amount of Bi, Lu or Ilu were successfully synthesized, and also indicate that the desired amounts of amine-functionalized Bi and Lu or ILu molecules could be introduced into dextran by our synthetic method (Table 1).

Table 1. Elemental analysis data of dextran-based CL probes.

Probe[a]	Found (%)				Calculated (%)				MW
	C	H	N	S	C	H	N	S	
Dex2000-$(Lu)_{2627}$-$(Bi)_{385}$	44.36	5.85	5.50	0.46	44.36	5.85	5.50	0.46	2701209
Dex500-$(Lu)_{559}$-$(Bi)_{34}$	43.62	5.85	4.39	0.17	43.60	5.85	4.39	0.17	631556
Dex2000-$(ILu)_{3262}$-$(Bi)_{275}$	43.69	5.65	5.96	0.31	43.69	5.65	5.96	0.31	2837018
Dex500-$(ILu)_{321}$-$(Bi)_{15}$	42.02	5.85	2.78	0.08					593056

[a]Dex2000 and Dex500 mean dextranT2000 and dextranT500, respectively.