培養した後、アンピシリン (50 μg/ml) を含んだ寒天培地で、37°C、一晩培養した。一晩培養したコロニーを液体培地に植菌し、37°C で一晩培養し、この培養液からプラスミドを QIA Spin Miniprep Kit を用いて調製した。このプラスミドに組み込んだ DNA の塩基配列は、BigDye Terminator 1.1 Cycle Sequencing Kit を用いて決定した。

3. CYP3A4 との結合検出

(A) HRP-SA による検出

ナイロン膜に CYP3A4 (2 μg/μl)を 1 μl と 2 μl ドットブロットし、ドライヤーで乾燥後、膜をスキムミルク 50 mg を含む PBS 2 ml に 30 分間浸し、ブロッキングした。膜を PBS 2 ml で 3 回洗浄した後、一本鎖または二本鎖ビオチン化 DNA 10 μg を含む TE buffer 1.5 ml 中、室温で 3 時間反応させた。反応後、膜を TE buffer 1.5 ml で 5 分間 3 回洗浄し、市販の horse radish peroxidase-streptoavidine (HRP-SA) 3 μl を含む PBS 1.5 ml に浸し、室温で 30 分間反応させた。0.15% Triton X-100 を含む PBS 1.5 ml で 5 分間洗浄を 3 回行い、Lumi Glo kit を用いて化学発光させ CCD カメラにより画像検出した。

(B) TMPG による検出

ナイロン膜に CYP3A4 (2 μg/μl)を 1 μl と 2 μl ドットブロットし、ドライヤーで乾燥後、膜をスキムミルク 50 mg を含む PBS 2 ml に 30 分間浸し、ブロッキングした。膜を PBS 2 ml で 3 回洗浄した後、一本鎖または二本鎖ビオチン化 DNA 10 μg を含む TE buffer 1.5 ml 中で室温 3 時間反応させた。反応後、膜を TE buffer 1.5 ml で 5 分間 2 回、さらに水 1.5 ml で 5 分間 1 回洗浄した。洗浄した膜を、0.1 M tetra-*n*-propylammonium phosphate 溶液 (pH 8.5) に 10 秒間、30 mM TMPG 溶液に 15 秒間浸した後、CCD カメラで 2 分間化学発光を画像検出した。

C. 研究結果

1. CYP と親和性のある DNA の分離と塩基配列の決定

59 mer DNA プールを材料として、CYP3A4 に結合する DNA の探索を試みた。DNA の分子量は約 18,000, CYP3A4 の分子量は約 66,000 なので、DNA の中に CYP3A4 と結合する分子が存在すれば、分子量の差により、結合型と非結合型の DNA を限界ろ過フィルターで分離できると考えた。研究方法 1 の操作を 10 サイクル行った後のポリアクリルアミドゲル電気泳動の結果を Fig. 2 に示す。

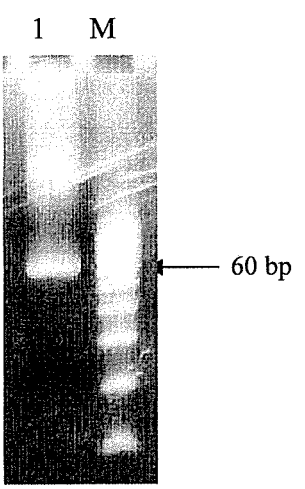

Fig. 2 PAGE of 10 cycle proceeding.
Lane 1 sample, Lane M marker DNA

このサンプルを pMOS Blue vector へ組み込み、大腸菌を形質転換したところ、約 200 個のコロニーが生育し、その中から無作為に 12 個のコロニーを選択した。これらからプラスミドを調製して、塩基配列を調べた結果、Table 1 に示す 9 つの配列が決定できた（数字はコロニーの番号を示す)。

Table 1 Nucleotide sequences obtained from cloning (5'→3')

1.	GGGACCGCAAACCAAGGGGGGGAGC
2.	GGGATTGAGGAGGGAGCGTTGGCAT
3.	GGGAGGGTAGCAGGCGANGGACCCA
4.	GGGACCAGGGGCCGTGGTGGTCAC
6.	GGGAGGAGATAGGGGGAGCGCCCGT
8.	GGGAAGTAGGCAGGAGTGTCCCGGGG
10.	GGGAGGGAGCGAAGTATTATTCCGT
11.	GGGACAGATGAAAGGCGGATAGTTA
12.	GGGACAGATGAAAGNCGGNTAGTTA

赤い文字は、5 つ以上の配列で共通の塩基を示している。これらの中で、コロニーNo.11 と 12 の配列はほぼ同じであった。

全ての DNA において GGGA という配列が同じ位置に存在しているが、5'末側プライマー配列の直後であり、GGGA 周辺の 5'末側プライマー配列を含めた配列が CYP との結合に重要な可能性がある。

2. CYP との結合性の評価

1 の結果から、コロニーNo.11 と 12 の配列がほぼ同じであったことから、No.11 について CYP3A4 との結合性を調べた。まず、No.11 のプラスミドを鋳型にして、PCR を行い、二本鎖ビオチン化 DNA を増幅した。この二本鎖ビオチン化 DNA からアビジンカラムにより、一本鎖ビオチン化 DNA を調製した。ビオチン化 DNA の濃度は、260 nm での吸光度を測定することより算出した。この一本鎖および二本鎖ビオチン化 DNA を用いて、ナイロン膜上の CYP3A4 を HRP-SA により検出した結果を Fig. 3 に示す。

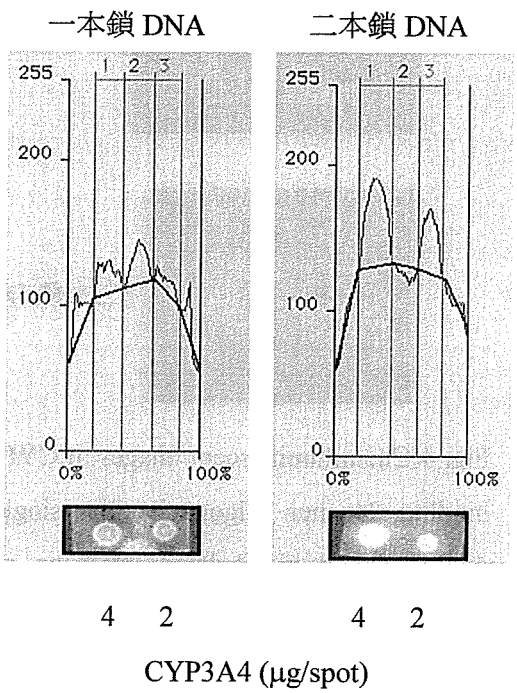

Fig. 3 Chemiluminescent images of CYP3A4 binding single or double strand biotinylated DNA on nylon membrane.

Fig. 3 に示すように、ナイロン膜上で、No.11 の一本鎖および二本鎖ビオチン化 DNA を用いて CYP3A4 を検出することができた。しかし、アプタマーの検索は、

一本鎖 DNA の状態で行っていたので、一本鎖 DNA が強く結合すると予想していたが、二本鎖 DNA の方が強く結合していた。これに関しては、原因は不明であるが、一本鎖よりも親和性がある二本鎖 DNA が得られたものと考えている。

次に、HRP-SA の代わりに TMPG 試薬[1] を用いて、CYP に結合した No.11 の一本鎖および二本鎖ビオチン化 DNA を直接化学発光検出した。結果を Fig. 4 に示す。

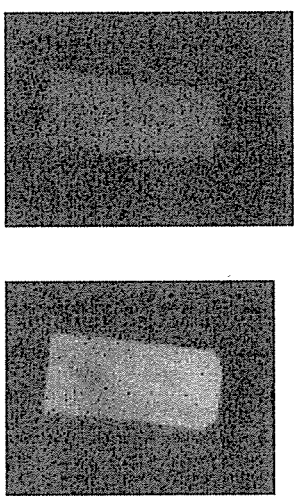

Fig. 4 Chemiluminescent images of CYP3A4 binding 24 mer oligonucleotide, single or double strand biotinylated DNA on nylon membrane.

TMPG 試薬により、CYP に結合した DNA の直接検出を行ったが、今回得られたビオチン化 DNA は検出できなかった。HRP-SA を使用した場合 (Fig. 3) と比較して、今回の方法では、TMPG 化学発光検出法の感度が十分ではなかったと考えられる。

D. 考察

標的分子に特異的に結合するアプタマーと呼ばれる核酸分子は、化学合成による大量生産が可能、抗体のような変性の心配がない、様々な物質（毒性物質や低分子物質など）を標的分子にできる、などの利点があり、抗体に替わるものとして期待されている。本研究では、テーラーメイド医療の指標となる CYP の選択的な高感度検出を目的として、59 mer の DNA プールから CYP3A4 と親和性を持つアプタマーの探索を、SELEX 法を参考に行った。得られた 9 種の DNA 全てにおいて GGGA という配列が同じ位置に存在しており、この GGGA 周辺の 5'末側プライマー配列を含めた配列が CYP との結合に重要な可能性がある。また、得られた配列中にはグアニンが多く存在し、これに関しては、他のタンパク質に対するアプタマーにおいて、guanine quartet conformation と呼ばれるグアニンに富む領域が重要であることが報告されている。

今回得た No.11 は、PCR による増幅後アビジンカラムにより精製していたが、実験に十分な量の DNA を得るのが困難であった。今後、収量を上げるための手法の改善が必要であり、さらに、結合定数なども求める必要がある。また、検出方法なども含めて、No.11 以外のサンプルについても結合定数や特異性などを調べる予定である。また、No.11 の CYP に対する結合性は、一本鎖より二本鎖の方が高いものであった。これまで報告されて

いるアプタマーは、ほぼ全て一本鎖のDNA またはRNA であり、二本鎖についてはあまり調べられていない。仮に二本鎖DNA アプタマーが開発できれば、RNAや一本鎖DNA に比べて、PCR により増幅後すぐに使用でき、非常に安定、取り扱いも容易であるという利点がある。さらに、Fig. 1 に示したスクリーニングにおいても、一本鎖にする工程が省略できるため、今後、二本鎖アプタマーの検索も同様に行う予定である。

また、これらDNA アプタマーとは別に、RNA アプタマーの探索も試みる必要がある。RNA は、DNA に比べて極めて不安定であるが、複雑な二次、三次構造をとることができ、一般的にDNA よりも高親和性であるため、これまでに多くのRNA アプタマーが報告されている。今回の方法により、CYP に親和性を持つDNA 分子を得られたことから、同様の方法でRNA アプタマーを収得できると期待できる。

今回得られたDNA アプタマーのCYP 分子種に対する特異性は検討していないが、今後、CYP 分子種に対する特異性も調べる予定である。このようなCYP 分子種特異的なアプタマーが得られれば、発光性の高分子プローブを用いることによって、CYP の特異的な検出法の構築が期待できる。

E. 結論

CYP に対するDNA アプタマーの探索を行ったところ、CYP に結合する分子が得られた。今回行った方法を改良することで、さらに高親和性のアプタマーが得られるものと期待できる。

F. 研究発表

1. 論文発表

(1)K. Tonooka, T. Kabashima, M. Yamasuji, and M. Kai. Facile determination of DNA-binding nuclear factor-κB by chemiluminescence detection. Anal. Biochem. 364, P30–36 (2007)

(2) K. Ito, Y. Nakajima, Y. Xu, N. Yamada, Y. Onohara, T. Ito, F. Matsubara, T. Kabashima, K. Nakayama, and T. Yoshimoto. Crystal structure and mechanism of tripeptidyl activity of prolyl tripeptidyl aminopeptidase from *Porphyromonas gingivalis*. J. Mol. Biol. 362, P228-240 (2006)

(3) 甲斐雅亮, 椛島 力：ベーシック分析化学（第 13 章 質量分析, 第 16 章 タンパク質と核酸の標識）; ㈱化学同人, 東京, pp. 169-185, pp. 210-222 (2006).

2. 学会発表

(1) 殿岡恵子、太田和子、椛島 力、甲斐雅亮：TMPG 試薬によるDNA 結合タンパク質の高感度簡易化学発光検出；日本薬学会第 126 年会 講演要旨集 (2) P36 仙台 (2006)

(2) 伊藤佳代、椛島 力、太田和子、甲斐雅亮：糖質応答領域結合タンパク質の変異体の機能解析；日本薬学会第 126 年会講演要旨集 (2) P36 仙台 (2006)

(3) Moses N.Wainaina、太田和子、椛島　力、甲斐雅亮：Sensitization of amino acids derivatives using 4-cyanoisoindolyl aniline in the post cleavage conversion of peptide for protein sequence；日本薬学会第 126 年会講演要旨集（2）P36　仙台（2006）

(4) Huan Zhang、椛島　力、甲斐雅亮：Detection of CYP3A4 by chemiluminescent polymeric probes on PVDF membrane；生物発光化学発光研究会第 24 回学術講演会抄録集 P56-57　東京（2006）

(5) 殿岡恵子、椛島　力、甲斐雅亮：DNA 結合型 NF-κB の簡易化学発光検出；生物発光化学発光研究会第 24 回学術講演会抄録集 P58-59　東京（2006）

(6) Zhang Huan、椛島　力、甲斐雅亮：Chemiluminescent detection of cytochrome P450 by polymeric isoluminol probes；日本分析化学会第 55 年会　講演要旨集 P287　大阪（2006）

(7) Wainaina N.Moses,椛島力、甲斐雅亮：Fluorescent derivatization with CIA in an alternate post cleavage conversion of anilinothiazolinone derivatives for protein sequencing;日本分析化学会第 55 年会　講演要旨集 P287　大阪（2006）

(8) 殿岡恵子、椛島　力、甲斐雅亮：標識 DNA を必要としない DNA 結合タンパク質の発光検出；日本分析化学会第 55 年会講演要旨集 P288　大阪（2006）

(9) 殿岡恵子、椛島　力、甲斐雅亮：TMPG による溶液中 DNA の迅速な化学発光検出反応；第 23 回日本薬学会九州支部大会講演要旨集 P130　熊本（2006）

(10) 山筋睦美、椛島　力、甲斐雅亮：TMPG 反応の PVDF 膜上での核酸化学発光検出；第 23 回日本薬学会九州支部大会講演要旨集 P131　熊本（2006）

(11) 古村匡崇、椛島　力、甲斐雅亮：ルミノールデキストランプローブによるナイロン膜上の DNA 化学発光画像検出；第 23 回日本薬学会九州支部大会　講演要旨集 P132　熊本（2006）

(12) Huan Zhang、椛島　力、甲斐雅亮：Application of dextran luminol probe on cytochrome p450 detection；第 23 回日本薬学会九州支部大会　講演要旨集 P133　熊本（2006）

(13) Moses N.Wainaina, Tsutomu Kabashima, Masaaki Kai：Sequencing of native insulin by HPLC derivatized with fluorescent CIA in alternative post cleavage conversion；第 23 回日本薬学会九州支部大会　講演要旨集 P134　熊本（2006）

G. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Keiko Tonooka, Tsutomu Kabashima, Mutsumi Yamasuji, Masaaki Kai	Facile determination of DNA-binding nuclear factor-κB by chemiluminescence detection	Anal. Biochem.	364	30-36	2007
Huan Zhang, Chaivat Smanmoo, Tsutomu Kabashima, Jianzhong Lu, Masaaki Kai	Dextran-Based Polymeric Chemiluminescent Compounds for the Sensitive Optical Imaging of a Cytochrome P450 Protein on a Solid-Phase Membrane	Anal. Chem.		投稿中	2007 予定
Masaaki Kai, Takayuki Shibata, Kouichi Ebisu, Tsutomu Kabashima, Myung Koo Lee, David M. Williams	Sensitive chemiluminescence-imaging detection of telomere DNA by use of luminol-modified macromolecular probe	Nucleic Acid Res.		投稿中	2007 予定
Tsutomu Kabashima, Zhiqiang Yu, Yoshiki Nakagawa, Takayuki Shibata, Jianzhong Lu, Masaaki Kai	A Selective Fluorescence Reaction for Peptides and Chromatographic Analysis	Anal. Chem.		投稿中	2007 予定
Moses N. Wainaina, Takayuki Shibata, Chaivat Smanmoo, Tsutomu Kabashima, Masaaki Kai	Fluorescence detection of amino acids in the post-cleavage conversions for peptide Sequencing	Anal. Biochem.		投稿中	2007 予定

研究成果の刊行物・別刷

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Facile determination of DNA-binding nuclear factor-κB by chemiluminescence detection

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Abstract

A simple, rapid, and sensitive method for the assay of a sequence-specific DNA-binding protein, nuclear factor-κB (NF-κB), has been developed by using a DNA-detectable chemiluminogenic reagent and a centrifugal filter that distinguishes different molecular sizes. After the formation of a complex between NF-κB and DNA, the unbound DNA is separated from the complex by the centrifugal filter. The amount of the bound NF-κB is estimated by chemiluminescence detection of the bound DNA. This detection is performed within 2 min at room temperature by the use of a chemiluminogenic reagent, 3′,4′,5′-trimethoxyphenylglyoxal, which selectively recognizes guanine moiety in oligonucleotides or DNAs. This method does not require any labeled probes or antibodies and can determine a concentration as low as 5 nM of DNA-binding NF-κB. The sensitivity is nearly the same as that of other methods such as gel shift assay using fluorescence-labeled probes and enzyme-linked immunosorbent assay. Therefore, the current method provides a convenient tool for surveying various DNA-binding proteins.

Keywords: NF-κB; Chemiluminescence detection; DNA-binding protein; TMPG

Sequence-specific DNA-binding proteins play critical roles in the biology of cells. These proteins are involved in the regulation of gene transcription and DNA replication, recombination, repair, and restriction [1–4]. Among the various sequence-specific DNA-binding proteins, transcription factors have been estimated to constitute 6 to 7% of all proteins expressed by eukaryotic genomes, which are responsible for the transfer of biological information from genes to proteins [5,6]. These transcription factors have become potential targets in medical diagnosis and drug development because of their pivotal roles in the pathways and networks involving the regulation of gene expression [7].

A prominent protocol for the determination of various sequence-specific DNA-binding proteins has attracted considerable attention from many research fields such as genomics and proteomics. However, the conventional methods for the detection of those proteins, including gel shift assay, DNA footprinting assay, and enzyme-linked immunosorbent assay (ELISA[1]), usually are time-consuming. In addition, they require labeled probes and/or specific antibodies for the detection of the DNA-binding protein in combination with electrophoresis for separation.

In this article, we describe a convenient method for the determination of sequence-specific DNA-binding nuclear factor-κB (NF-κB). NF-κB is a ubiquitous redox-sensitive transcription factor that responds to pro-inflammation

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[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; TMPG, 3′,4′,5′-trimethoxyphenylglyoxal; dsDNA, double-stranded DNA; SDS, sodium dodecyl sulfate; ChRE, carbohydrate response element; dI–dC, poly(deoxyinosinic deoxycytidylic) acid; NMWL, nominal molecular weight limit; TPA, tetra-*n*-propyl ammonium phosphate; DMF, *N,N*-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; DMSO, dimethyl sulfoxide; MW, molecular weight; BSA, bovine serum albumin; FRET, fluorescence resonance energy transfer.

caused by cytokines and oxidative stress [8,9]. By the activation with tumor necrosis factor-α (TNF-α), NF-κB translocates into the nucleus, where it binds to specific nucleotide sequences [8,9]. The cellular response of NF-κB to inflammatory and stress signals has been implicated in disease conditions such as atherosclerosis, cancer, diabetes, and Alzheimer's disease [10]. For the sensitive and facile detection of NF-κB bound to DNA, we employed 3′,4′,5′-trimethoxyphenylglyoxal (TMPG) as the chemiluminogenic reagent. TMPG reacts specifically with guanine moiety in DNA to form a chemiluminescent product [11]. The reaction conditions were first improved for the rapid detection of DNA with TMPG within 2 min in a neutral medium, and then a facile method was developed for the quantitative and sensitive determination of the DNA-binding NF-κB by the chemiluminescent detection with the TMPG reaction.

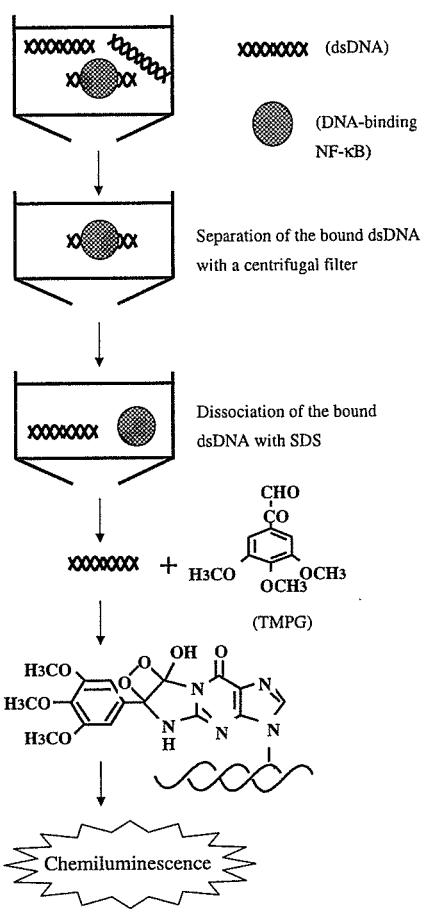

Fig. 1. Schematic protocol for the assay of DNA-binding NF-κB by means of chemiluminescence reaction with TMPG.

Fig. 1 illustrates our protocol proposed for the determination of the DNA-binding NF-κB. In this assay, the bound and unbound double-stranded DNAs (dsDNAs) that contain 14 guanine bases after the formation of a complex between NF-κB and dsDNA were readily separated with a centrifugal filter that was able to remove molecular sizes lower than 100,000 Da. The bound dsDNA, which was dissociated with sodium dodecyl sulfate (SDS), was filtrated. The amount of the DNA in the filtrate was then detected by the chemiluminescence reaction with TMPG. The proposed method does not require any labeled probes or specific antibodies, and it allows the analysis of the interaction between DNAs and proteins by using conventional inexpensive instruments.

Materials and methods

Materials and reagents

Purified recombinant human NF-κB p50 was purchased from Promega (Madison, WI, USA). A dsDNA, dsDNA/NF-κB with the sequences 5′-AGTTGAGGGGACTTTCCCAACTAGGAATCT-3′ and 3′-TCAACTCCCCTGAAAGGGTTGATCCTTAGA-5′, was used for binding to the protein, NF-κB. The underlined sequence represents the protein-binding site [8]. Another dsDNA, dsDNA/ChRE with the sequences 5′-GGGCGCACGTGGCACTCACGTGGTTCC-3′ and 3′-CCCGCGTGCACCGTGAGTGCACCAAGG-5′ (which binds to a carbohydrate response element (ChRE)-binding protein [12]), was used as the negative control for dsDNA/NF-κB. Those single-stranded oligonucleotides and a 60-mer oligonucleotides, 5′-$(TTAGGG)_{10}$-3′, were products obtained from Sigma–Genosys Japan (Ishikari, Japan). Poly(deoxyinosinic deoxycytidylic) acid (dI–dC) was purchased from Roche (Mannheim, Germany) and was used to suppress nonspecific binding of the protein. Nuclear extracts of HeLa cells were purchased from Active Motif (Carlsbad, CA, USA). The concentration of whole proteins in each extract was 2.5 g/L based on information provided by the supplier. Centrifugal filter devices (Ultra-free MC 100,000 nominal molecular weight limit [NMWL] filter units) were purchased from Millipore (Bedford, MA, USA).

TMPG was synthesized according to previously reported conditions [13], but with a slight modification as follows. To a stirred solution of selenium dioxide (45 mmol) in dioxane (40 ml) was added 3,4,5-trimethoxyacetophenone (50 mmol) at 40 °C. The mixture was refluxed for 2 h, and selenium dioxide (45 mmol) was then added to the reaction mixture. After being refluxed for 3 h, the mixture was filtrated to remove an insoluble selenium. The filtrate was mixed with 240 ml of H_2O and then kept at 4 °C for approximately 15 h. The formed precipitates were recrystallized from water to give colorless needles (melting point 101–102 °C, yield 60–70%).

Chemiluminescence reaction of DNA with TMPG

The TMPG reaction was carried out as follows. A portion (20 μl) of a sample in H_2O was mixed with 10 μl of 0.1 M tetra-*n*-propyl ammonium phosphate (TPA) solution (pH 7.0) in a glass tube, and then a 200-μl portion of 10 mM TMPG in *N,N*-dimethylformamide (DMF) was added to start the chemiluminescence reaction. Chemiluminescent intensity was measured with a luminescence reader (BLR-201, Aloka, Tokyo, Japan) for 2 min at room temperature (24–26 °C) after the addition of TMPG.

Assay of DNA-binding NF-κB

A single-stranded oligonucleotide and its complementary oligonucleotide were mixed at an equimolar concentration (4.0 μM each) to form dsDNA. The mixture was heated at 95 °C for 5 min and cooled slowly to room temperature. NF-κB (0–100 nM) was mixed with 1.0 μM dsDNA/NF-κB and 0.25 g/L dI–dC in 40 μl of a binding buffer (10 mM Tris–HCl [pH 7.5], 0.5 mM ethylenediaminetetraacetic acid [EDTA], 0.5 mM dithiothreitol, and 10% glycerol) and then reacted at room temperature for 30 min.

The reaction mixture (20 μl) was transferred to a centrifugal filter device and centrifuged at 2000 *g* for 2 min to separate free dsDNA from NF-κB-binding dsDNA. The filter was washed twice with 100 μl of the binding buffer containing 0.01% Triton X-100 by centrifugation at 2000 *g* for 10 min. A portion (40 μl) of 0.1% SDS solution was added to the filter device, which was then incubated at 37 °C for 10 min. The filter device was centrifuged at 4000 *g* for 10 min, and a 20 μl portion of the filtrate was used to react with TMPG to determine the amount of DNA-binding NF-κB.

DNA-binding NF-κB in nuclear extracts of HeLa cells, which were incubated in advance for 30 min in the presence or absence of 20 ng/ml TNF-α, was determined as described above. For this sample preparation, the extract solution containing 2.5 g/L whole proteins was diluted with H_2O to adjust the protein concentration to 0.31 g/L in the binding reaction mixture (40 μl).

Gel shift assay

A gel shift assay was performed as described previously [12] by using fluorescein isothiocyanate (FITC)-labeled oligonucleotides. NF-κB (0–50 nM) was mixed with 250 nM FITC-labeled dsDNA/NF-κB and reacted in the binding buffer. The reaction mixture (20 μl) was loaded onto a 4.5% polyacrylamide gel (75 × 90 mm), and then free dsDNA and dsDNA-binding NF-κB in the mixture were separated by electrophoresis at 60 V for approximately 3 h. The separated components on the gel were detected by a fluorescence imaging analyzer (FLA5000, Fujifilm, Tokyo, Japan).

Results and discussion

Chemiluminescence reaction of DNA with TMPG

We previously reported that TMPG could react with guanine moieties in a DNA molecule to form a product that emits chemiluminescence [11]. However, a long incubation time (20 min) in a medium containing sodium phosphate buffer (pH 6.5) and dimethyl sulfoxide (DMSO) is required to produce the chemiluminescent product. To develop a more facile procedure, we optimized the conditions of the TMPG reaction by using DMF instead of DMSO and using a tetra-alkyl ammonium salt instead of sodium phosphate buffer (pH 6.5). As a result, we found that DMF and TPA at pH 7.0 were effective not only for rapid formation of the chemiluminescent product but also for fast emission of the chemiluminescence.

Fig. 2A shows the emission profile of the chemiluminescence produced from dsDNA/NF-κB depending on the time after mixing with TMPG in the presence of DMF and TPA. The chemiluminescence reached the maximum at 20 s and then rapidly decreased, and no chemiluminescence was observed in the absence of dsDNA. There was a good correlation between the chemiluminescent intensity and the amount of dsDNA/NF-κB that contains 14 guanine bases in the molecule. dsDNA/NF-κB at 5 nM could be detected within 2 min by the modified reaction conditions with TMPG. This TMPG reaction did not give any signal for proteins and oligonucleotides such as dI–dC because there is no guanine moiety in its molecule and thus it permitted the selective detection of guanine-containing DNAs and oligonucleotides such as 5′-$(TTAGGG)_{10}$-3′, as shown in Fig. 2B. There are 14, 19, and 30 guanine moieties in dsDNA/NF-κB, dsDNA/ChRE, and 5′-$(TTAGGG)_{10}$-3′, respectively. Compared with the chemiluminescent intensity from these guanine-containing DNAs, the intensity was enhanced according to the number of guanine bases. There was a good relationship between chemiluminescent intensity and the number of guanine bases, although the intensity was not exactly proportional to guanine contents. Therefore, the lower limit of detection was dependent on the guanine contents in the DNA molecule. The results indicate that the TMPG reaction is preferable for the sensitive detection of specific protein-binding DNA containing many guanine bases in the molecule, and thus dI–dC is usable as a suppressor for the nonspecific binding of protein to DNA.

Assay of DNA-binding NF-κB

To verify the proposed assay (Fig. 1), we first prepared dsDNA/NF-κB (double-stranded molecular weight [MW] 18,400 Da) that can bind specifically to the dimer proteins of NF-κB (dimer MW 98,800 Da) [8–10]. We also used dsDNA/ChRE (double-stranded MW 16,600 Da) that binds specifically to a ChRE protein [12] as the negative control. The NF-κB protein at various concentrations

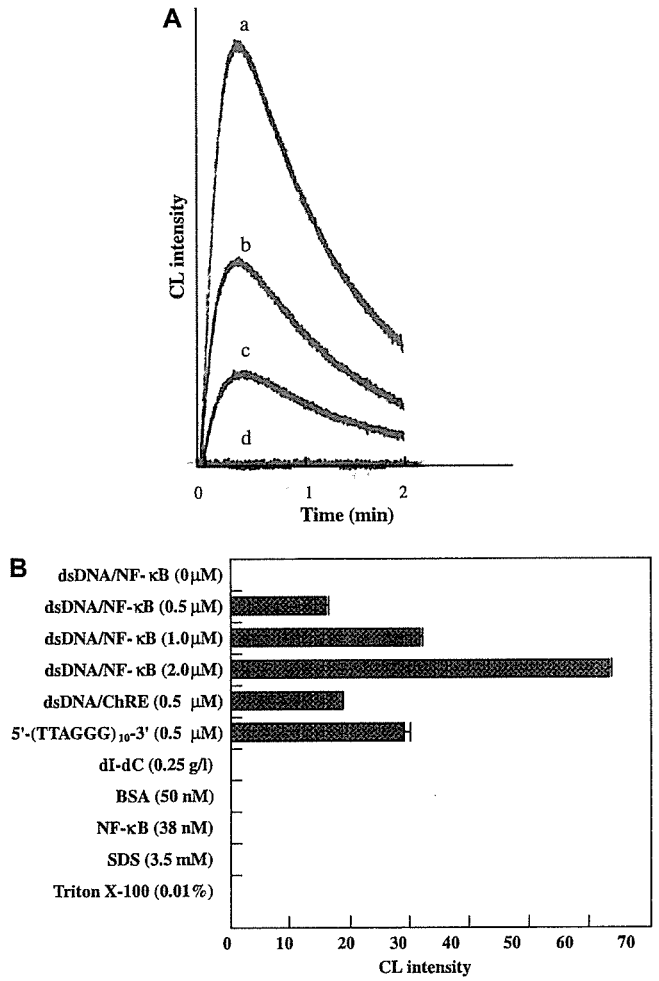

Fig. 2. Chemiluminescence (CL) reactions of DNAs with TMPG. (A) Different concentrations of dsDNA/NF-κB at 2.0 μM (a), 1.0 μM (b), 0.5 μM (c), and 0 μM (d) were reacted individually with TMPG, and their chemiluminescent emissions were recorded for 2 min. (B) dsDNAs, oligonucleotides, and proteins were reacted individually with TMPG, and their total chemiluminescent intensities for 2 min were measured ($n = 3$ each).

was reacted individually with 1.0 μM dsDNA/NF-κB. After the separation of the unbound dsDNA/NF-κB in the reaction mixture by a centrifugal filter device, the dsDNA bound to NF-κB was released from the complex in the filter device. This centrifugal filter device was equipped with an ultrafiltration membrane for the removal of particulates less than MW 100,000 Da. After the centrifugation, free dsDNA in the filtrate was detected by the TMPG reaction.

The chemiluminescent intensity from the bound dsDNA/NF-κB was increased by increasing the concentration of NF-κB (Fig. 3). The dissociation of the bound dsDNA from the DNA–protein complex was performed by SDS treatment. SDS at a concentration of 0.5 to 0.05% (w/v) gave a maximum and constant intensity of chemiluminescence due to the free dsDNA. However, the chemiluminescent intensity was decreased drastically by the use of 1.0% SDS because SDS at a concentration greater than 1.0% inhibited the TMPG reaction. On the other hand, when water was used instead of SDS solution for the dissociation, the chemiluminescent intensity from the free dsDNA could not be observed. These results indicate that 0.1% SDS solution could quantitatively dissociate the bound dsDNA from the DNA–protein complex. The calibration graph showed a linear relation between the chemiluminescent intensity and the concentration of 6.3 to 50 nM NF-κB, indicating a linear correlation coefficient (r^2) of 0.997; the linear equation was $y = 0.117x + 1.72$, with x and y indicating the concentration of NF-κB and the intensity, respectively. The lower limit of detection

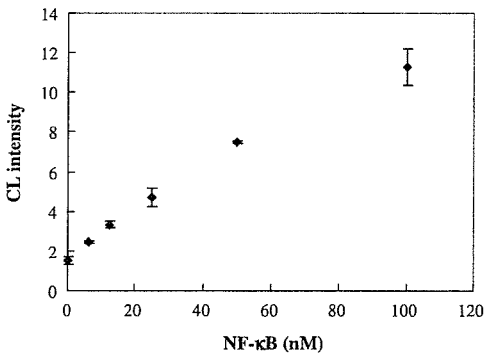

Fig. 3. Calibration curve for the protein of NF-κB bound to dsDNA/NF-κB. Various concentrations of NF-κB (0, 6.3, 12.5, 25, 50, and 100 nM) were reacted individually with 1.0 μM dsDNA/NF-κB in a 40 μl binding reaction mixture ($n = 4$ each). CL, chemiluminescence.

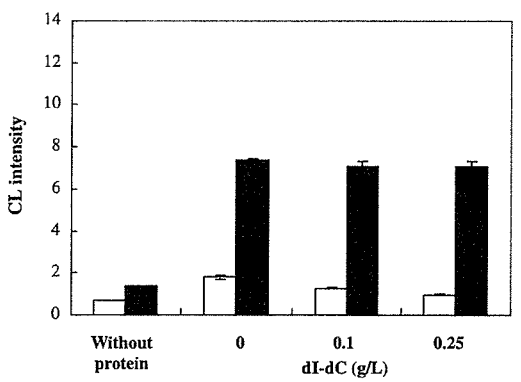

Fig. 4. Effect of dI–dC on the specific binding of NF-κB to dsDNA. NF-κB (50 nM) was reacted with 1.0 μM dsDNA/ChRE (white column) or 1.0 μM dsDNA/NF-κB (black column) in the presence of dI–dC (0, 0.1, or 0.25 g/L) in a 40-μl binding reaction mixture ($n = 4$ each). The left column indicates that the buffer was used instead of NF-κB as a blank for the binding reaction mixture containing 0.25 g/L dI–dC. CL, chemiluminescence.

was approximately 5 nM for NF-κB. The results indicate that the DNA-binding protein was determined sensitively by the proposed assay.

Specificity and assay of DNA-binding NF-κB in nuclear extracts

To evaluate the specific binding of NF-κB to dsDNA/NF-κB, dsDNA/ChRE was used instead of dsDNA/NF-κB for the binding reaction. The dsDNA/ChRE contains an E-box sequence (CACGGG) [14] that corresponds to the consensus binding site for other transcription factors such as upstream stimulatory factors and their related family members [15]. When dsDNA/ChRE was reacted with NF-κB, the chemiluminescent intensity was nearly the same as that obtained without protein (Fig. 4). This result indicates that most of the NF-κB protein did not bind to dsDNA/ChRE. In addition, the chemiluminescent intensity due to the nonspecific binding dsDNA/ChRE decreased in the presence of dI–dC. Therefore, we added 0.25 g/L dI–dC to the reaction mixture so as to suppress the nonspecific binding of NF-κB to other dsDNA. In this case, chemiluminescent intensity due to the specific binding dsDNA/NF-κB did not change in the presence or absence of dI–dC (Fig. 4).

When bovine serum albumin (BSA), instead of NF-κB, was mixed with dsDNA/NF-κB, the chemiluminescent intensity due to the nonspecific binding dsDNA/NF-κB indicated the background value that was obtained without the protein (Fig. 5). These results indicate that the proposed protocol has quite high reliability for the determination of sequence-specific DNA-binding NF-κB.

Conventional methods, such as gel shift assays [16], DNA footprinting [17,18], and ELISA [19], have been used for the detection of specific interaction between DNA and protein in crude nuclear extracts. This prompted us to investigate the applicability of the current method to the determination of DNA-binding NF-κB in a crude nuclear extract of cultured cells. For the assay of the target NF-κB in the nuclear extract of HeLa cells, HeLa cells were stimulated either with or without 20 ng/ml TNF-α. It has been reported that the concentration of NF-κB in the nucleus is induced by the TNF-α stimulation [20]. As shown in Fig. 5, the concentration of DNA-binding NF-κB in the nuclear extract after the stimulation with TNF-α was approximately four times greater than that without the stimulation. According to the calibration curve in Fig. 3, the concentrations of NF-κB in the stimulated and nonstimulated nuclear extracts were estimated as 78 and 20 nM, respectively.

Comparison with gel shift assay

Gel shift assay is one of the most useful methods for the detection of the DNA-binding proteins [16]. Therefore, our assay was compared with the gel shift assay. FITC-labeled dsDNA/NF-κB was used for the gel shift assay. As shown in Fig. 6, 12.5 nM NF-κB was detected at a lower limit of detection by the gel shift assay, although this electrophoresis took approximately 3 h for the assay. By the gel shift assay using radiolabeled DNA probes, the detection limit of NF-κB may be approximately 4 nM based on information provided by the supplier. Thus, the sensitivity of our method is comparable to that of other conventional methods [16–18].

Conclusions

The proposed method was applied to the facile and rapid assay of the DNA-binding NF-κB in crude nuclear extracts of mammalian cells, although similar DNA-binding proteins cannot be distinguished by this method. To

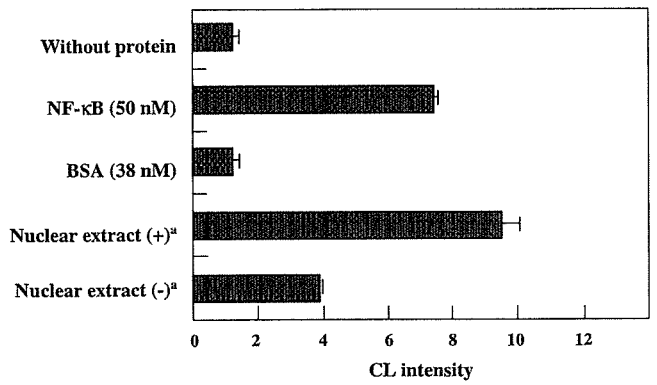

Fig. 5. Assay of DNA-binding NF-κB by the current method. NF-κB (50 nM), BSA (38 nM), and nuclear extracts of HeLa cells were reacted individually with 1.0 μM dsDNA/NF-κB in a 40-μl reaction mixture ($n = 4$ each), and the concentrations of the bound NF-κB were determined by chemiluminescence (CL) detection. [a]HeLa cells were incubated with (+) or without (−) TNF-α before extraction.

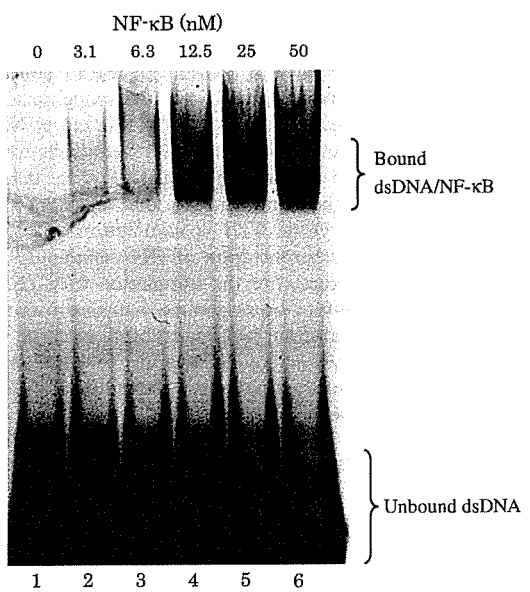

Fig. 6. Fluorescence detection of NF-κB bound to dsDNA/NF-κB by a gel shift assay. The concentrations of NF-κB used for the binding reaction (40 μl) were 0 nM (lane 1), 3.1 nM (lane 2), 6.3 nM (lane 3), 12.5 nM (lane 4), 25 nM (lane 5), and 50 nM (lane 6).

establish the conditions for the proposed method, we examined the reaction of DNA with TMPG and found that TPA and DMF enhanced the progress of the reaction between DNA and TMPG. This TMPG reaction progressed at room temperature and provided strong chemiluminescence within 2 min for guanine-containing DNA. For the assay of the sequence-specific DNA-binding NF-κB, we employed two surface-active agents: Triton X-100 and SDS. Triton X-100 was used to remove the unbound dsDNA from a centrifugal filter, whereas SDS was used to dissociate the bound dsDNA from the DNA–protein complex. We also employed dI–dC to suppress the nonspecific binding of NF-κB to dsDNA. dI–dC at a high concentration could suppress the nonspecific binding without any influence for the detection of the specific binding. Using these reagents, we succeeded in determining the target protein, NF-κB, in a crude nuclear extract of cultured cells.

The detection sensitivity of our method is nearly the same as that of the gel shift assay using a fluorescent probe. In addition, our method does not require any labeled probes or antibodies and can be performed within 1 h for many samples, whereas the gel shift assay and ELISA require several hours. However, the gel shift assay can distinguish more precisely the molecular size of a DNA–protein complex. Recently, several fluorescence resonance energy transfer (FRET) probes have been used for the detection of DNA-binding proteins [21]. Using this method, it is generally difficult to design and prepare the FRET probe, although the method is simple, rapid, and sensitive. The current method may detect many samples at the same time because the simultaneous detection is possible by the use of a microtiter plate. Therefore, this method will be a convenient tool not only for the detection of various DNA-binding proteins but also for the analysis of unknown transcription factors in mammalian cells.

Acknowledgments

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References

[1] C.O. Pabo, R.T. Sauer, Transcription factors: Structural families and principles of DNA recognition, Annu. Rev. Biochem. 61 (1992) 1053–1095.

[2] A. Pingoud, A. Jeltsch, Recognition and cleavage of DNA by type-II restriction endonucleases, Eur. J. Biochem. 246 (1997) 1–22.
[3] N.L. Craig, The mechanism of conservative site-specific recombination, Annu. Rev. Genet. 22 (1988) 77–105.
[4] C. Margulies, J.M. Kaguni, Ordered and sequential binding of DNA protein to oriC, the chromosomal origin of *Escherichia coli*, J. Biol. Chem. 271 (1996) 17035–17040.
[5] N.M. Luscombe, S.E. Austin, H.M. Berman, J.M. Thornton, An overview of the structures of protein–DNA complexes, Genome Biol 1 (2001) 1–37.
[6] S. Jones, J.M. Thornton, Protein–DNA interactions: The story so far and a new method for prediction, Comp. Funct. Genom. 4 (2003) 428–431.
[7] P.P. Pandolfi, Transcription therapy for cancer, Oncogene 20 (2001) 3116–3127.
[8] P.J. Barnes, M. Karin, Nuclear factor-κB: A pivotal transcription factor in chronic inflammatory diseases, N. Engl. J. Med. 336 (1997) 1066–1071.
[9] L.I. McKay, J.A. Cidlowski, Molecular control of immune/inflammatory responses: Interactions between nuclear factor-κB and steroid receptor-signaling pathways, Endocrinol. Rev. 20 (1999) 435–459.
[10] Y. Yamamoto, R.B. Gaynor, Role of the NF-κB pathway in the pathogenesis of human disease states, Curr. Mol. Med. 3 (2001) 287–296.
[11] M. Kai, S. Kishida, K. Sakai, A chemiluminescence derivatization method for detecting nucleic acids and DNA probes using a trimethoxyphenylglyoxal reagent that recognizes guanine, Anal. Chim. Acta 381 (1999) 155–163.
[12] T. Kawaguchi, M. Takenoshita, T. Kabashima, K. Uyeda, Glucose and cAMP regulate the l-type pyruvate kinase gene by phosphorylation/dephosphorylation of the carbohydrate response element binding protein, Proc. Natl Acad. Sci. USA 98 (2001) 13710–13715.
[13] E. Kojima, Y. Ohba, M. Kai, Y. Ohkura, Phenylglyoxal and glyoxal as fluorogenic reagents selective for N-terminal tryptophan-containing peptides, Anal. Chim. Acta 280 (1993) 157–162.
[14] E.N. Kaytor, H. Shih, H.C. Towle, Carbohydrate regulation of hepatic gene expression: Evidence against a role for the upstream stimulatory factor, J. Biol. Chem. 272 (1997) 7525–7531.
[15] H. Yamashita, M. Takenoshita, M. Sakurai, R.K. Bruick, W.J. Henzel, W. Shillinglaw, D. Arnot, K. Uyeda, A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver, Proc. Natl. Acad. Sci. USA 98 (2001) 9116–9121.
[16] M.G. Fried, D.M. Crothers, Equilibria and kinetics of lac repressor–operator interactions by polyacrylamide gel electrophoresis, Nucleic Acids Res. 9 (1981) 6505–6525.
[17] D.J. Galas, A. Schmitz, DNase footprinting: A simple method for the detection of protein–DNA binding specificity, Nucleic Acids Res. 5 (1978) 3157–3170.
[18] A.M. Benotmane, M.F. Hoylaerts, D. Collen, A. Belayew, Nonisotopic quantitative analysis of protein–DNA interactions at equilibrium, Anal. Biochem. 250 (1997) 181–185.
[19] D. Shalloway, T. Kleinberger, D.M. Livingston, Mapping of SV 40 DNA replication origin region binding sites for the SV 40 DNA replication antigen by protection against exonuclease III digestion, Cell 20 (1980) 411–422.
[20] J.A. DiDonato, M. Hayakawa, D.M. Rothwarf, E. Zandi, M. Karin, A cytokine-responsive IκB kinase that activates the transcription factor NF-κB, Nature 388 (1997) 548–554.
[21] J. Wang, T. Li, X. Guo, Z. Lu, Exonuclease III protection assay with FRET probe for detecting DNA-binding proteins, Nucleic Acids Res 33 (2005) e23.

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) imaging is a promising detection system that is increasingly used for an ultra-sensitive quantification and localization of analytes in a wide range of applications. Herein we report a simple method to synthesize four dextran-based polymeric CL compounds and then employ them for the sensitive CL imaging of a cytochrome P450 (CYP) protein on a polyvinyliden difluoride (PVDF) membrane. These dextran-based polymeric CL compounds were tethered with a small amount of biotin linker and a large number of CL emitter luminol (or isoluminol). The initially poor CL intensity was found to be subsequently improved with our currently developed acetonitrile-tetraalkylammonium hydroxide-H_2O_2 system. Its CL intensity was further increased by employing 0.45 mM Fe (III) as a catalyst. The addition of avidin led to the formation of an extend-framework polymeric dextran probe, and this polymeric dextran probe subsequently enhanced the CL intensity due to an increasing number of luminol (or isoluminol) units. We successfully applied this probe for an immunoblotting assay of CYP3A4. This newly developed CL probe provides one of the simple and promising techniques for CL imaging.

Chemiluminescence (CL) has been exploited within a wide range of applications in various fields such as clinical, medical, pharmaceutical, biotechnological, molecular biological and environmental analyses due to its high sensitivity, high selectivity, and simple instrumentation.[1-3] In particular, luminol and isoluminol are well-known for their CL property. Their mechanistic details have been well exemplified. In order to obtain a good signal strength and high sensitivity, extensive works on the synthesis of luminol (or isoluminol) derivatives and the searching for a novel enzymatic or non-enzymatic catalyst are desirable.[4-15] Most researches on this field mainly focused on the flow injection analysis, high performance liquid chromatography analysis and capillary electrophoresis.[4-15]

CL imaging represents a promising detection system that is increasingly used for an ultra-sensitive quantification and localization of several analytes in a wide range of applications. Currently, microarray technology has gained its popularity for the analysis of biological samples due to its benefits in that simultaneous detection of multiple analytes, less required sample and reagents, and high output of test results are possible.[16,17] Standard methods that are currently available for reading out microarrays are mainly based on luminescence-imaging techniques. A CL signal is measured by a charge-coupled device (CCD) camera and then quantified by imaging software. For CL imaging, a traditional CL method usually employed horseradish peroxidase (HRP) as a labeling reagent, although its less stability at the room temperature and a high background interference limit its applicability in a clinical analysis, especially in serum samples.[18, 19] Thus, the demand for a rapid and a sensitive detection of biological samples have been increased due to an increasing amount of biological samples and a minute amount of samples available for an analysis. Especially, the development of a non-enzymatic CL reagent for CL imaging is encouraging. In addition, avidin-biotin interaction has been well recognized in immuohistochemistry, enzyme-linked immunosorbent assay (ELISA), and molecular biology.[20-22] Avidin is an egg-white derived glycoprotein with a high affinity for biotin. Avidin consists of four identical subunits which each subunit serves as a single binding site for biotin. The affinity of biotin binding to avidin is extremely high with a binding (association) constant of 10^{15} M^{-1}. The biotin

molecule can easily be activated and coupled to either antigens or antibodies with a completed retention of its activity. Considering of these advantages, it would be of our interests to develop a CL probe tethered with biotin. Using avidin is a key molecule in order to extend the structural framework of biotin-related CL reagent.

Herein, we describe a simple and efficient method for the synthesis of four dextran-based polymeric CL compounds which tethered with a biotin linker in a small amount and CL emitter luminol (or isoluminol) in a large amount. The addition of avidin forms polymeric dextran CL probe. The resultant polymeric dextran probe enhanced the CL intensity due to an increased luminol (or isoluminol) units. We succeeded to employ this probe for the sensitive CL imaging of CYP3A4 protein on a PVDF membrane (Figure 1).

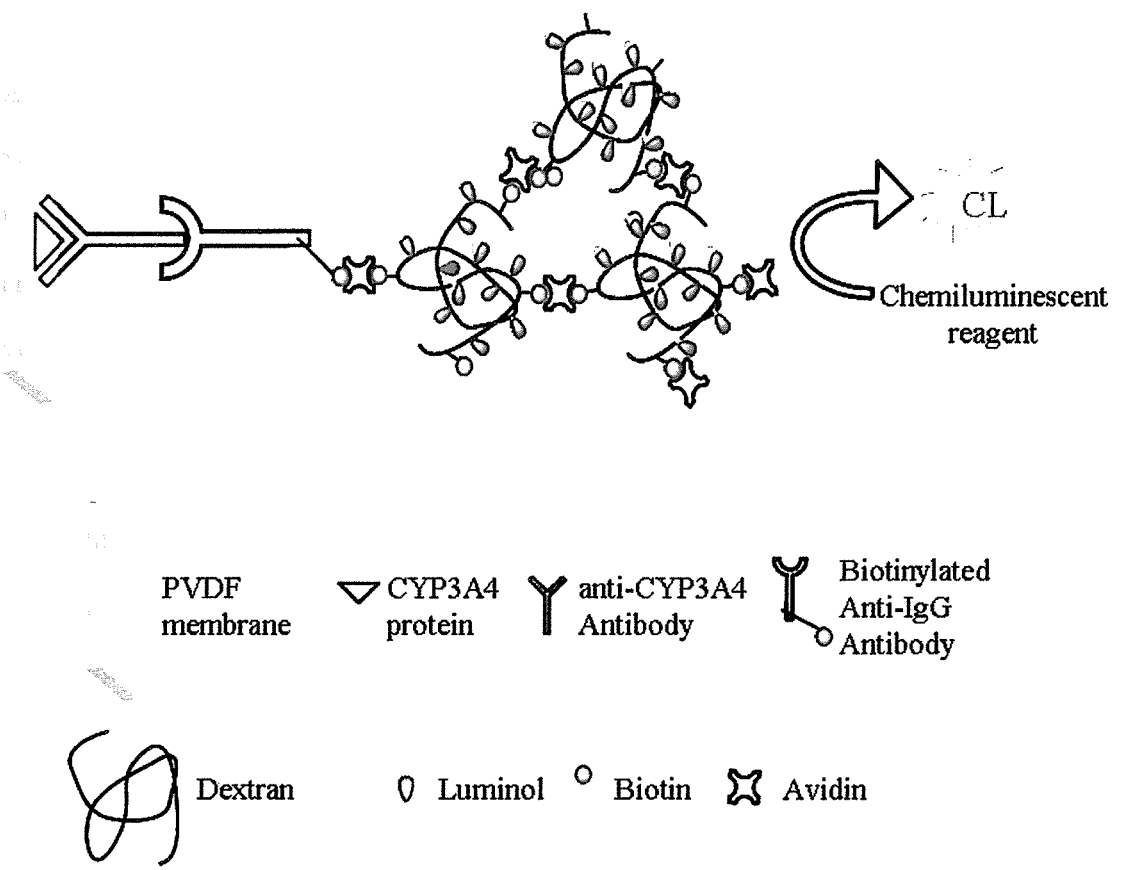

Figure 1. Detection of CYP3A4 on a PVDF membrane using a polymeric dextran-based CL probe.

EXPERIMENT SECTION

Materials and Reagents. Dextran T2000 (average MW 2 x 10^6 D) and T500 (average MW 5 x 10^5 D) were purchased from Pharmacia Fine Chemicals (Sweden). $NaIO_4$ and avidin were purchased from Wako (Japan). 6-Hydrazidohexyl D-biotinamide was obtained from Dojindo (Japan), and isoluminol, luminol and $NaBH_4$ were purchased from Nacalai Tesque (Japan). Nylon membrane and Polyvinyliden Difluoride (PVDF) membrane (0.2-μm pore size) were supplied by ATTO (Japan). Tetrabutylammonium hydroxide (TBA) and tetrapropylammonium hydroxide (TPA) were supplied by Sigma (USA). Recombinant human CYP3A4 protein and anti-human CYP rabbit polyclonal antibody were purchased from Oxford Biomedical Research (USA). Biotinylated anti-rabbit IgG goat antibody was supplied by Beckman (USA).

Synthesis of Dextran-Based Polymeric CL Compounds (Figure 2). Dextran (T2000 or T500) 400 mg was dissolved in 80 or 30 mL of Milli-Q water, followed by precipitation with 300 mL of methanol. The precipitated dextran was re-dissolved in 60 or 30 mL of water and then mixed with sodium periodate (317 mg, 1.48 mmol).[23] The oxidation of dextran was controlled and monitored with UV-spectrophotometer at 310 nm. After approximate 30% oxidation, the oxidized dextran was precipitated with 400 mL of methanol, and then dissolved in 80 mL of DMSO. To this reaction mixture, 6-hydrazidohexyl D-biotinamide (30 mg, 0.08 mmol) was added and allowed to stir at room temperature for 3 h. Glacial acetic acid (16 mL) and luminol (or isoluminol) (240 mg, 1.35 mmoL) were subsequently added into the reaction mixture and left stirring at 60 ℃ overnight. The modified dextran was precipitated with 300 mL of methanol followed by dissolving the modified dextran in 30 mL of ethylene glycol. Sodium borohydride (870 mg, 23 mmol) was subsequently added into the reaction mixture and left stirring at room temperature for 4 h. The resultant biotin and luminol (or isoluminol)-containing dextran was precipitated with 300 mL of methanol. The precipitate was dissolved in 10 mL of Milli-Q water followed by re-precipitation with 300 mL of methanol. The dextran-based polymeric CL compound was then dried *in vacuo* and its purity was checked by gel-filtration liquid chromatography (GFLC). The GFLC analysis was carried out using a TSK gel T2000SW column

(Tosoho, Japan). Elemental analysis for the synthesized compound was analyzed by Tokyo Chemical Industry (Japan).

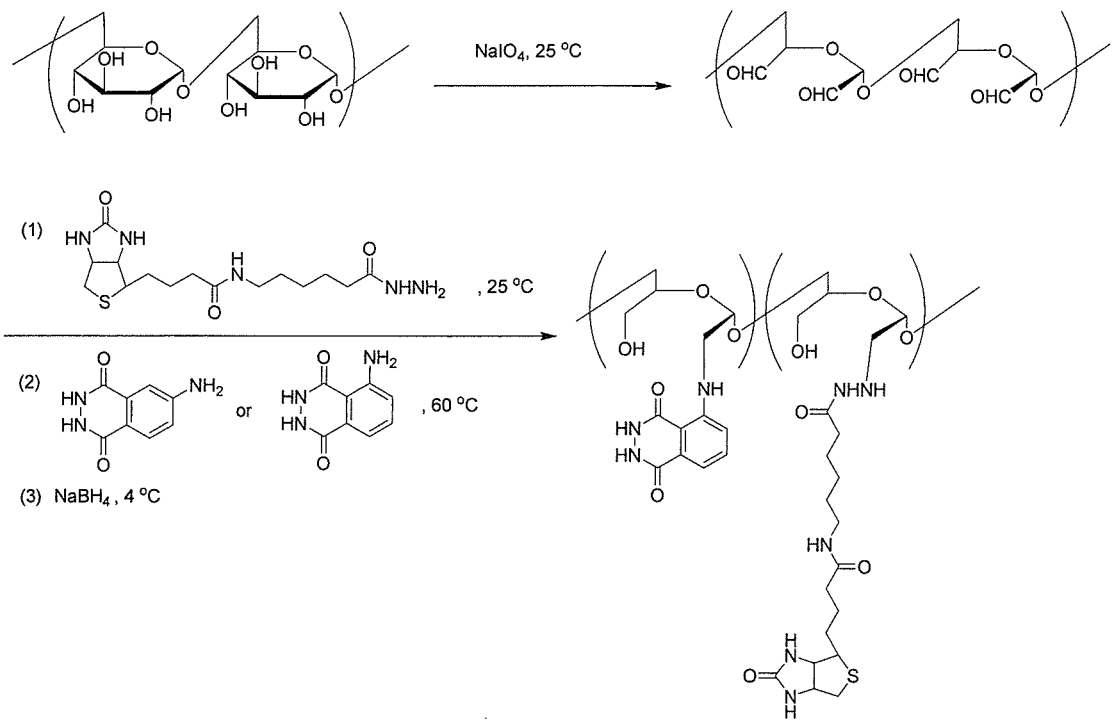

Figure 2. The synthesis of dextran-based polymeric CL compound.

CL Imaging of Dextran-Based Polymeric CL Compounds on Nylon Membrane. Different concentrations of dextran-based polymeric CL compounds (2 μL each) were spotted on a nylon membrane and dried *in vacuo* for 10 min. The nylon membrane was then washed with 2 mL of methanol at 37 ℃ for 5 min. The membrane was then immersed into a CL reagent (CH_3CN 700 μL, 0.5 M TBA 300 μL, 30% H_2O_2 50 μL) for 10 s before the CL detection for 2.0 min with CCD camera or alternatively used another CL reagent (CH_3CN 700 μL, 1.0 M TPA 300 μL, 30% H_2O_2 50 μL, 10 mM $FeCl_3$ 50 μL) for 3 s before the CL detection for 1.0 min with CCD camera. The CL intensity was measured by Light Capature AE-6971/2 (ATTO, Japan).

Evaluation of Binding Constant Between Avidin and Dextran-Based Polymeric CL Compounds. A PVDF membrane was spotted with 2 μL of 99.5 % ethanol followed by an avidin aqueous solution

(0.5 nmol/2 μL). The corresponding membrane was dried *in vacuo* for 10 min before incubation with different concentrations (0.05 mg/mL, 0.075 mg/mL, 0.1 mg/mL and 0.15 mg/mL) of a dextran-based polymeric CL compound in 2 mL of 10 mM, pH 7.4 phosphate buffered saline (PBS) at 37 ℃ for 30 min. The membrane was then washed with a mixture (4 mL) of 0.15% (v/v) triton X-100 and 10 mM PBS at 37 ℃ (×2) followed by washing with 2 mL of 75 % methanol. The membrane was dried at 37 ℃ for 10 min *in vacuo* before immersing into the CL reagent (CH_3CN 700 μL, 0.5 M TBA 300 μL, 30% H_2O_2 50 μL) for 10 s before the CL detection for 2.0 min with CCD camera. Using Scatchard-plot method, the binding constant K of probe to avidin and the molar binding ratio of avidin to the dextran-based polymeric CL compound were obtained.

Detection of CYP3A4 on PVDF Membrane by Using Dextran-Based Polymeric CL Compound (Figure 1). A PVDF membrane was spotted with 2 μL of 99.5 % ethanol followed by spotting 2 μL each of 190 fmol, 380 fmol, 760 fmol, 1500 fmol and 3000 fmol human recombinant CYP3A4 protein. After drying the membrane at 37 ℃ for 10 min *in vacuo*, the membrane was incubated with 5.7 μg/mL anti-human CYP3A4 rabbit polyclonal antibody in 2 mL of 10 mM PBS at 37℃ for 60 min. The membrane was then washed with 3 mL of 10mM PBS at 37 ℃ for 10 min (×2) and further incubated with 8.0 μg/mL biotinylated anti-rabbit IgG goat antibody in 2 mL of 10 mM PBS at 37 ℃ for 40 min. The membrane was washed with 3 mL of 10mM PBS (×2) before incubation at 37 ℃ for 30 min with the polymeric CL probe. The luminol-biotin-dextranT500 compound (2 mg) was pre-incubated with 2 mg avidin and 10 mg BSA in 2 mL of 10 mM PBS at 37 ℃ for 1 h. The membrane was then washed with 3 mL of a mixture of 0.15 % triton X-100 and 10 mM PBS solution (×2) followed by washing with 2 mL of 75 % methanol. The membrane was dried at 37 ℃ for 10 min *in vacuo*. The membrane was then immersed into a CL reagent (CH_3CN 700 μL and 1.0 M TPA 300 μL) 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 the CL detection for 1.0 min with CCD camera.