reading or sophisticated instrumentation such as CCD and photon-counting cameras for processing image data. In addition, the luminescent reagents are considerably more stable than radioisotopes, and adaptable for detecting hybridization on membranes or with immobilized DNA.

The chemiluminescent derivatizing method for detecting DNA and d(G)n on a nylon membrane, based on the chemical reaction with TMPG, has the advantages of speed and simplicity for the quantitative determination of nucleic acids and DNA probes containing d(G)n. Using a d(G)n probe enhances the chemiluminescence in proportion to the amount of guanylic acid in the probe molecule. The TMPG reaction is useful not only for in situ detection of guanine-rich regions of the genome, but also for immobilized hybridization assays.

Current methods for DNA detection usually require enzymatic amplification of the target DNA sequence prior to analysis. For example, the PCR technique selectively increases the concentration of the target sequence relative to unrelated sequences. PCR methods, however, introduce ambiguities resulting from contamination by different DNA sequence. Therefore, a definitive method is required for the analysis of a single, original DNA sequence. To achieve this objective, the sensitivity and speed of the chemiluminescent enhancement techniques described in this chapter must be improved.

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A Chemiluminescence Reaction between Hydrogen Peroxide and Acetonitrile and Its Applications

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Here we report a new chemiluminescence reaction between basic aqueous H_2O_2 and acetonitrile. Its ultraweak chemiluminescence could be greatly enhanced by luminol, isoluminol-labeled streptavidin, and an Edman-type fluorescent reagent. Light emission was intense and long-lived, and this facilitated the initiation of the reaction and the measurement of the light emission. The present results permit us to propose a series of convenient, highly sensitive, and enzyme-free techniques for the detection and quantification of luminol, related conjugates, acetonitrile, and amino acids. Overall, this new chemiluminescence reaction will be quite promising for numerous applications in immunoassay, DNA hybridization, environmental monitoring, and postcolumn chromatographic detection.

Chemiluminescence (CL) has been exploited with a wide range of applications in different fields such as biotechnology, pharmacology, molecular biology, and clinical and environmental chemistries.[1-9] The most frequently used CL reactions are luminol–peroxidase and 3-(2′-spiroadmantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane (AMPPD)–alkaline phosphatase systems. These CL reactions offer the possibility of a high sensitivity and a lasting light signal, making more accurate measurements possible and simplifying the measurement procedure. However, the use of enzyme can be problematic, as enzymes may be unstable or cause background interference especially in serum samples as a result of hemoglobin release during red blood cell lysis. Thus, it is a great challenge for the chemists to develop new CL reactions with wide applications, though it would also be important to extend the applications of classical CL reactions.

Here we report such a new CL reaction between basic aqueous hydrogen peroxide and acetonitrile. Although this reaction itself only emitted an ultraweak CL, the light could be greatly enhanced by luminol, related conjugates, and our recently developed Edman-type fluorescent reagent, 4-(2′-cyanoisoindonyl)phenylisothiocyanate (CIPIC).[10] In addition to obtaining a high sensitivity, a longer light signal was observed for routine applications especially with a simple setup. Moreover, the mixing of CL reagents could be generally performed outside the measuring device, making convenient and reproducible CL measurements possible. Based on this fact, the potential use of this reaction was demonstrated in the fields of labeled protein quantification, organic solvent monitoring, and postcolumn amino acid detection.

EXPERIMENTAL SECTION

Materials. Luminol and isoluminol-labeled streptavidin were obtained from Sigma. Acetonitrile, propanenitrile, benzonitrile, and other organic compounds were purchased from Wako. CIPIC was synthesized as described.[10]

Batch CL Measurements. Light-producing reactions were carried out in 12 × 75 mm disposable culture tubes containing 10 μL of different concentrations of luminol or isoluminol-labeled streptavidin or CIPIC. A total of 100 μL of 0.1 M Na_2CO_3 and 20 μL of 0.1 M H_2O_2 were added. Then 20 or 50 μL of distilled water was added to adjust the total volume, and the tubes were placed in the luminescence reader (BLR-201, Aloka). The 50 or 20 μL of acetonitrile was injected, and the cover was closed to initiate CL. The signal was displayed and integrated for a 10-s interval, in arbitrary units. Kinetics of the CL was monitored on a recorder connected to the luminescence reader.

HPLC Conditions. The HPLC system consisted of a Tosoh (Tokyo, Japan) HLC-803D high-performance liquid chromatograph, with a Rheodyne model 7125 syringe-loading sample injector (100-μL loop). A reversed-phase column of TSKgel ODS-80T$_M$ (150 × 4.6 mm i.d., particle size 5 μm) (Tosoh) was used. The column temperature was ambient (25 ± 4 °C). The separation of the CIPIC-conjugated amino acids was carried out with a mobile phase of acetonitrile (45–90%) and 0.1 M acetic acid–triethylamine (pH 5.0), and the flow rate was set at 0.5 mL/min. The column eluate was introduced into the postcolumn reactor. In the system, 1.0 M H_2O_2 and 0.1 M sodium borate buffer (pH 11) were added to the eluate stream with two reagent-delivery pumps each at a flow rate of 0.23 mL/min. CL was monitored by intelligent CL detector (Jasco 825-CL).

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10.1021/ac010688d CCC: $20.00 © 2001 American Chemical Society
Published on Web 11/09/2001

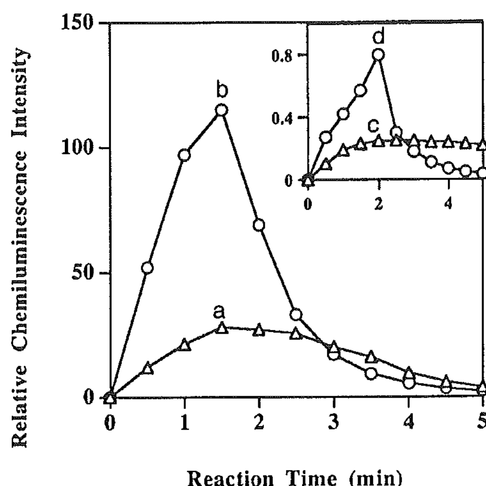

Figure 1. Time course of the kinetic profile of the CL reaction in (a) 5 and (b) 25% acetonitrile and 1 pmol/tube luminol. Inset: (c) 5and (d) 25% acetonitrile alone (0.01 M H_2O_2 and 0.05 M Na_2CO_3).

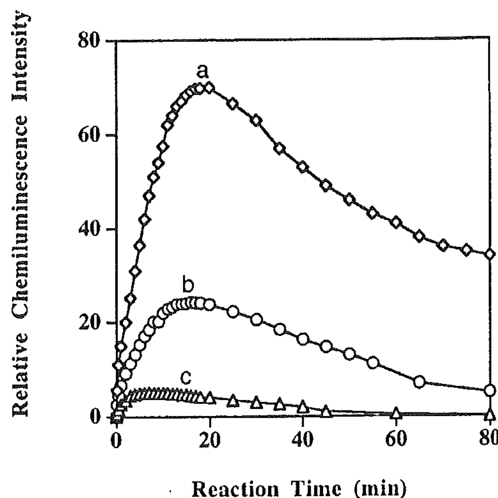

Figure 2. Time course of the kinetic profile of the luminol-enhanced CL reaction; luminol concentration: (a) 100 pmol; (b) 10 pmol; (c) 1 pmol/tube (1% acetonitrile, 0.01 M H_2O_2, and 0.05 M Na_2CO_3).

Derivatization Procedure. A 50-μL aliquot of amino acid solution (acetonitrile–water, 1:4, v/v) was mixed with 100 μL of 10 mM CIPIC and 50 μL of pyridine. The mixture was heated at 80 °C for 15 min and then cooled to room temperature. A 200-μL aliquot of 50 mM phosphate buffer (pH 8.5) and chloroform were added, respectively. After centrifugation, 100 μL of supernatant was transferred to 50 μL of 2.5 M HCl solution and the mixture was heated at 80 °C for 15 min. A 25-μL aliquot of 2.5 M sodium carbonate was added into the mixture after cooling to the room temperature. An aliquot (50–100 μL) was used as the sample for HPLC.

RESULTS AND DISCUSSION

An ultraweak CL was observed after mixing basic aqueous H_2O_2 and acetonitrile. CL was greatly enhanced by luminol and related conjugates. The kinetic profile of this new CL reaction is shown in Figure 1. With a 5% concentration of acetonitrile, the maximum peak was obtained at 1.5 min after mixing various reagents and then the signal slowly decreased (Figure 1a). This characteristic is useful for developing convenient and highly reproducible CL assays, since this longer lived signal over minutes facilitates the handling of CL measurements. In contrast, most of enzyme-free CL reactions reported previously have spontaneous emission and decay, which is difficult for routine bioanalytical applications.[11,12] In this case, the initiation generally must be performed in situ within the measuring chamber of the CL measuring device due to its short-lived signal over a few seconds. Otherwise, a poor reproducibility or low sensitivity is obtained. Furthermore, the signal increased by further increasing the amount of acetonitrile. The shape of the kinetic profile (Figure 1b) was essentially similar to that seen at 5% acetonitrile (Figure 1a), but the reaction proceeded with a relatively fast decay. Interestingly, the shapes of the kinetic profile were quite different when the amount of acetonitrile was decreased to 1% (Figure 2). After reaching the maximum peak, the signal decreased very slowly. For instance, with 100 pmol/tube luminol, the signal was still greater than 50% of peak height even after 80 min. This method may be useful in other fields, such as lightstick chemistry.

The effects of H_2O_2 and pH on the CL intensity were further examined. The intensity increased when the concentration of H_2O_2 was increased from 0 to 0.005 M. At a H_2O_2 concentration of higher than 0.01 M, the signal slowly decreased. The maximum intensity was observed in $NaHCO_3$–Na_2CO_3 buffer at a pH above 11. Therefore, the following experiments were done in the presence of 0.01 M H_2O_2 and 0.05 M Na_2CO_3.

Many investigations have confirmed that the CO_3^{2-} ion was a luminous species when exposed to a strong oxidant in a basic solution.[13] However, in our study, a signal was also observed in other buffers, such as borate–NaOH and NaH_2PO_4–NaOH. Although the signal was comparatively smaller than the signal observed in $NaHCO_3$–Na_2CO_3 buffer, this confirmed that CO_3^{2-} ion did not play a major role in this CL reaction.

Although it is well known that luminol emits a strong chemiluminescence in pure dimethyl sulfoxide (DMSO) or dimethylformamide (DMF) in combination with a strong base,[9] no CL was observed under the same experimental conditions by using other organic compounds, such as DMSO, DMF, ethanol, and methanol instead of acetonitrile. However, CL was also seen in the presence of propanenitrile or benzonitrile (Figure 3). Thus, this indicates that the nitrile group is a reactive part in the present CL reactions.[14] CL intensities in these reactions were in the order of acetonitrile ~ propanenitrile > benzonitrile, possibly because the CL reaction occurs by nucleophilic attack on a slightly higher electrophilic carbon of the nitrile group with less steric hindrance in the acetonitrile or propanenitrile, compared with that in the benzonitrile. Furthermore, ~32% CL intensity in the absence or presence of luminol was inhibited by the addition of 0.05 M sodium azide, which is claimed to be a quencher of single oxygen.[15] Other singlet oxygen scavengers were also very effec-

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Figure 3. Time course of the kinetic profile of the luminol enhanced CL reaction in 10% different solvent: (a) acetonitrile; (b) propanenitrile; (c) benzonitrile (luminol concentration, 1 pmol/tube).

Figure 4. Standard curves of luminol in different concentrations of acetonitrile: (a) 25%; (b) 10%.

Scheme 1

$$R-C{\equiv}N + {}^{\cdot}O-OH \rightarrow R-C(-O-OH){=}N^{\cdot} \xrightarrow{H_2O}$$

$$R-C(-O-OH){=}NH \rightarrow R-C(-O-OH){=}NH + H_2O_2 \rightarrow R-C(=O)-NH_2 + H_2O + {}^1O_2$$

$${}^1O_2 + {}^1O_2 \rightarrow ({}^1O_2)_2^{\cdot} \rightarrow 2{}^1O_2 + \textbf{Light}$$

$$\text{Luminol} + {}^1O_2 \rightarrow \text{Luminol}^* + \textbf{Light}$$

tive; the addition of 0.05 M dimethylfuran quenched 56% of the original signal and the addition of 0.05 M 1,4-diazabicyclo[2.2.2]-octane inhibited 68% of the original intensity. Therefore, it is believed that the CL was primarily caused by single oxygen. The CL spectrum of H_2O_2–acetonitrile–luminol was measured and compared with that of a conventional H_2O_2–HRP–luminol reaction. The results showed that both CL spectra were identical, with a maximum wavelength at about 425 nm. This may indicate that the intermediate of excited luminol produced in the presence of acetonitrile is the same as that described previously.[16,17]

Based on the above results, the mechanism can be summarized as shown in Scheme 1; singlet oxygen was produced and CL was induced with a longer lived and intense signal. In contrast, by using hypochlorite and H_2O_2 to generate singlet oxygen for the fluorescein CL reaction,[18] the CL reaction was emitted with a short-lived signal over a few seconds. By using photosensitization to prepare singlet oxygen for the CL reaction with *trans*-1-(2′-methoxyvinyl)pyrene,[19] an external light source was needed and currently it is somewhat difficult to employ it in the immunoassay and microplate detection. With this new reaction, the standard curves of luminol at 10 and 25% acetonitrile are shown in Figure 4. The measurable range at 10% acetonitrile was 10 pmol–100 fmol/tube, and the working range at 25% acetonitrile was 10 pmol–10 fmol/tube. The relative standard deviation was 4.6% at 100 fmol/tube luminol. With our simple setup, luminol was detectable at concentration as low as 1 fmol/tube. This demonstrated sensitivity was comparable to that of the most sensitive existing luminol CL methods,[20,21] with potential for far greater sensitivity.

It has been well documented that streptavidin can be used as a universal marker for different kinds of assays.[9] As a second application, we illustrated our new reaction for the detection of this commercial, labeled product. The measurable range was 1.0 μg–0.2 ng/tube, and as low as 200 pg/tube (~3 fmol/tube) streptavidin labeled with isoluminol was detectable for this commercial protein. According to Sigma, only 3.5 isoluminol molecules were attached to 1 streptavidin molecule. Many researchers indicated that the increase of the number of labels per protein was associated with an increase in sensitivity.[22] Diamandis et al. incorporated 14 dye molecules onto 1 streptavidin with direct labeling[23] and ~150 dye molecules/streptavidin through a polymeric backbone,[24] leading to a dramatic enhancement of the sensitivity. Therefore, with our new CL reaction, it can be expected that the detection limit of 200 pg/tube labeled streptavidin can be further improved by using indirect labeling through microsphere,[25] polymeric backbone,[24] and even liposome.[26]

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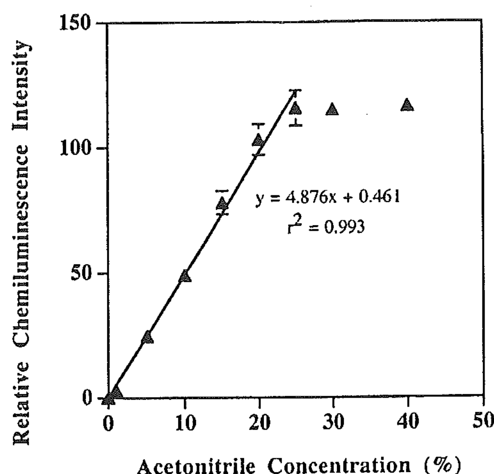

Figure 5. Standard curve of acetonitrile by using the luminol-enhanced CL reaction.

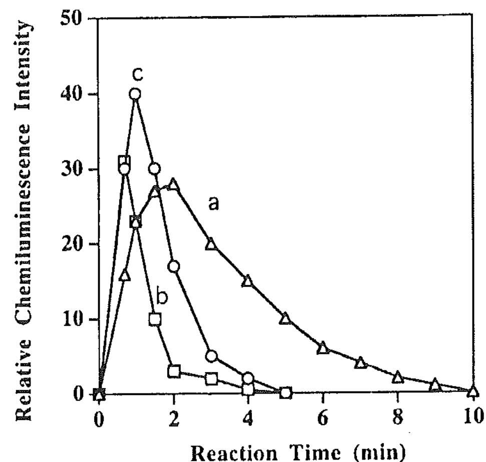

Figure 6. Time course of the kinetic profile of the ClPIC-enhanced CL reaction in (a) pH 9.0, (b) pH 9.6, and (c) pH 11.5 borate buffer (60% acetonitrile, 0.5 nmol/tube ClPIC, and 0.24 M H_2O_2).

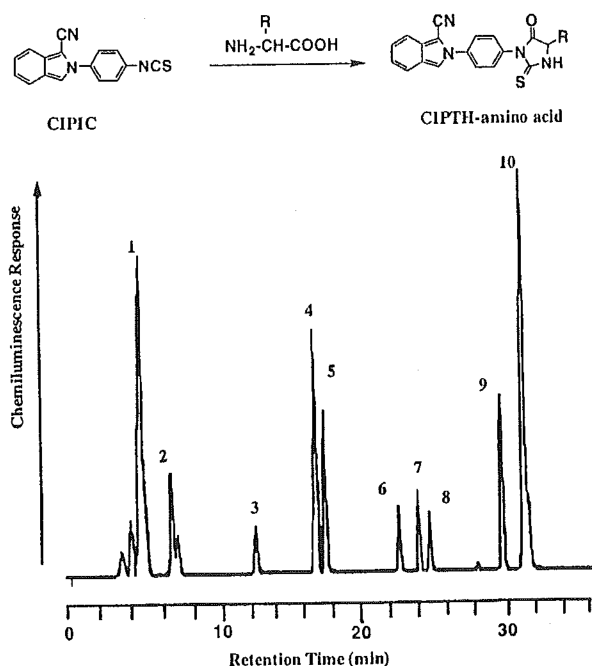

Figure 7. CL chromatogram of six amino acids, Peaks: 1, thiocarbomoyl amino acid residue; 2, α–NH_2-ClPTH-lysine; 3, ClPTH-threonine; 4, byproduct from ClPIC; 5, ClPTH-alanine; 6, ClPTH-proline; 7, ClPTH-isoleucine; 8, ClPTH-phenylalanine; 9, ε-NH_2-ClPTH-lysine; 10, byproduct from ClPIC (3.5 nmol for lysine and 0.7 nmol for others).

The more widely used methods for the detection of organic solvents are gas chromatography (GC) or distillation where the density or refractometry is determined in a subsequently step.[27] For instance, acetonitrile was determined by capillary gas chromatography after direct injection of deproteinized serum.[28] This can be a lengthy procedure requiring frequent instrument calibration. Recently, guided microwave spectroscopy with multivariate detection has been tested for use in the determination of acetonitrile instead of GC.[29] The working range of acetonitrile was 5–30% and the detection limit was not reported. Besides, as a complementary method, CL was used for the assay of organic solvents in water by using a γ-Al_2O_3 layer as catalyst.[30] When a mixture of air and organic molecules vaporized from a solution that flowed around the layer, CL was emitted during a catalytic oxidation. As a third application, we demonstrated that the luminol-enhanced CL method could be used for the sensitive and selective detection of acetonitrile from other common organic solvents, i.e., DMF, DMSO, ethanol, and methanol. The linear range was 0–25% acetonitrile, with a detection limit of 0.25% acetonitrile in water (Figure 5). These results provide a simple way to monitor this commonly used organic compound.

Furthermore, we found that our recently developed Edman-type fluorescent reagent, ClPIC, could also enhance this new reaction. A typical CL emission profile with the reaction time is shown in Figure 6. The shape of the kinetic profile of this enhanced CL reaction altered as the pH was increased. At pH 9.0, the reaction proceeded more slowly with peak height at 2 min and a decay to background at 10 min. At pH 9.6 and 11.5, the kinetic profiles were more similar consisting of a rapid rise to peak height at 1 min followed by a rapid decay to background by 5 min. CL intensity was maximum at pH 9.6, and the borate buffer was the best one compared with other buffers. Moreover, CL intensity increased with the increase of acetonitrile or H_2O_2 concentration. As described previously, ClPIC could react with amino acids with a high efficiency.[10] Thus, as a fourth application, we developed a new amino acid detection method based on ClPIC-enhanced CL reaction between aqueous H_2O_2 and acetonitrile. It is well known that the separation technique plays a key role for the detection of different analytes in complex matrixes. High-performance liquid chromatography (HPLC) has currently become dominant as a potential tool for the separation and quantification of a wide range of analytes in different fields.[31–33]

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Acetonitrile is one of the best solvents and used frequently for reversed-phase HPLC.[34,35] Therefore, the CIPIC-enhanced CL reaction is wholly compatible with the acetonitrile-based reversed-phase separation technique. For the detection of CIPIC-conjugated amino acids, H_2O_2 solution and borate buffer were simply mixed with the separated eluates and CL was monitored on a recorder. As shown in Figure 7, six CIPTH-amino acids were eluted within 30 min and well separated from each other. Each CIPTH-amino acid afforded one peak, with the exception of CIPTH-lysine which gave two peaks. Peaks 1, 4, and 10 were also confirmed by directly injecting free CIPIC after derivatization or thiocarbomoyl amino acids into the column. Detection limits of these amino acids were 0.3−0.8 pmol/injection.

CONCLUSIONS

This work introduces a new CL reaction with wide applications. Not only was a longer light signal observed but also a high sensitivity could be obtained even with a simple and inexpensive setup. Moreover, CL measurement does not require in situ initiator addition, making more accurate measurements possible and facilitating the measurement procedure. Four analytical applications here reported are supposedly exhaustive enough to illustrate the wide versatility of this new CL reaction. Overall, this new CL reaction is expected to be a useful tool for numerous applications in immunoassay, DNA hybridization, environmental monitoring, and postcolumn chromatographic detection. Moreover, our current data have shown that it is very easy to couple this new reaction to CL imaging for the detection of protein on a microplate and membrane with a charge-coupled device. Besides, special care should be taken when these CL reactions are applied for the analysis of real samples. Since the intermediate singlet oxygen is susceptible to interferences, many classes of compounds have been reported either to quench or to react with singlet oxygen. We have already found that sodium azide, dimethylfuran, and 1,4-diazabicyclo[2.2.2]octane quenched CL, and fluorescein and some indole compounds competed with luminol or CIPIC to react with singlet oxygen. Currently, we are using the luminol-enhanced reaction for the detection of some diagnostically important serum hormones and cancer markers and employing the CIPIC-enhanced reaction for the amino terminal sequencing of peptides and proteins.

ACKNOWLEDGMENT

We gratefully acknowledge financial support from the Japan Society for the Promotion of Science.

Received for review June 19, 2001. Accepted September 23, 2001.

AC010688D

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Anal Bioanal Chem (2002) 374:1064–1068
DOI 10.1007/s00216-002-1586-2

ORIGINAL PAPER

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Controlled kinetics of non-enzymatic chemiluminescence reactions for simple imaging of DNA and protein

Received: 2 April 2002 / Revised: 2 August 2002 / Accepted: 27 August 2002 / Published online: 23 October 2002
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Abstract A robust and sensitive non-enzymatic chemiluminescence (CL) imaging method is presented. In the method a fast-emitting CL reaction is tuned to furnish a slower-emitting reaction suitable for simple CL imaging. Typically, non-enzymatic CL reactions between luminol or fluorescein and oxygen species generated by KCN as catalyst, were rather fast and unsuitable for CL imaging; the speed of the reactions could, however, be reduced substantially by changing KCN for CH_3CN or benzonitrile. Light emission from the tuned CL reaction was intense and long-lived, and even with a simple arrangement high sensitivity could be achieved. The maximum CL peak was reached after approximately 1.5 min in the presence of 25% acetonitrile, and as little as 16 fmol commercial isoluminol-labeled streptavidin was detected and visualized on either microplate or membrane. The approach was further illustrated by imaging of DNA on a membrane and of antibody on a microplate by use of biotin–streptavidin chemistry. Overall, this simple, economical, and sensitive CL imaging system is expected to be very useful in biochemical analysis, and greatly complements currently used enzyme-based CL imaging methods, especially in routine applications.

Keywords Chemiluminescence · Kinetics · Catalyst · Imaging · DNA · Protein

Introduction

Chemiluminescence (CL) imaging is a promising detection system for ultrasensitive, quantitative analysis and for visualization of the spatial distribution of biomolecules such as antigens, enzymes, antibodies, and DNA probes in microplates, membranes, tissues, or cells [1, 2, 3, 4, 5, 6, 7, 8]. The systems currently used are based on a few enzyme-based CL reactions, either alkaline phosphatase with phenyl phosphate 1,2-dioxetane derivatives, or horseradish peroxidase with luminol–acridan reagent. Although many CL reactions have been reported, most have a short-lived signal, complete within a few seconds (flash type) and are unsuitable for sensitive and reproducible determinations in CL imaging by use of a charge-coupled device (CCD) or film camera. Thus all the well-established CL imaging methods use relatively few enzyme-based CL reactions, because they have a longer-lived signal which lasts for minutes or hours (glow type). The problem related to the use of enzyme labels is the instability of the enzyme, and reduced spatial resolution in the location of the labeled probe, because the product of the enzyme could diffuse in the medium [9]. A suitable means of tuning many flash-type CL reactions into glow-type reactions with high sensitivity would be desirable for routine applications.

Initially, we focused on non-enzymatic luminol-type compound-based CL reactions. We were attracted to these reactions because luminol-type compounds are very common CL reagents widely used for many kinds of assay [10, 11, 12, 13]. Currently, non-enzymatic luminol reactions have been employed exclusively as flash-type labels. Although a sub-optimum reaction pH could usually be used to slow down the reaction, with the associated disadvantage of low sensitivity, this was not a suitable means of controlling CL kinetics for CL imaging in practical applications. Herein we report a favorable means of controlling of CL kinetics for simple CL imaging.

The non-enzymatic CL reaction between luminol and oxygen species generated by KCN as catalyst is rather fast even without H_2O_2; it was, however, found that use of CH_3CN or benzonitrile instead of KCN led to a glow-type CL kinetics. The maximum CL peak was reached after approximately 1.5 min in the presence of 25% acetonitrile, and thus this flash-type non-enzymatic CL reaction was tuned for simple CL imaging. Although it is well known that luminol emits strong chemiluminescence in

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pure dimethyl sulfoxide (DMSO) or dimethylformamide (DMF) in combination with a strong base, no CL was observed under the same experimental conditions after changing acetonitrile for DMSO, DMF, etc. [14]. We also demonstrated that as little as 16 fmol isoluminol-labeled streptavidin could be easily detected and visualized on a microplate or membrane. As a real application, DNA was imaged on a membrane and antibody was visualized on a microplate, by use of biotin-streptavidin chemistry. Overall, this simple, economical and sensitive CL imaging system is expected to be very useful in biochemical analysis, and might greatly complement currently used enzyme-based CL imaging methods especially in routine application.

Experimental

Chemicals

All chemicals were of analytical-reagent grade and were used as received. The water was prepared by use of Milli-XQ equipment. Luminol, fluorescein, and isoluminol-labeled streptavidin were obtained from Sigma. Acetonitrile, benzonitrile, and other organic compounds were purchased from Wako, Japan. Biotinylated DNA markers were purchased from Vector Laboratories. Biotinylated Rabbit IgG (whole Ab) from donkey was purchased from Amersham Pharmacia Biotech and Nunc-Immuno plate (MaxiSorp surface) was obtained from Nalge Nunc International, Denmark.

Apparatus

Batch CL measurements were acquired by use of a luminescence reader (BLR-201, Aloka, Japan). Imaging was achieved by means of a Lumino CCD AE-6930 densitograph (Atto, Tokyo, Japan) and the data were processed by a computer running Densitometer Analyst version 4.0 software.

Batch CL procedures

Batch CL reactions were performed in 12 mm×75 mm disposable culture tubes containing 10 μL of different concentrations of luminol, fluorescein, melatonin, or isoluminol labeled streptavidin. Na_2CO_3 (0.1 mol L^{-1}, 100 μL) and H_2O_2 (0.1 mol L^{-1}, 20 μL) were added. Distilled water (20 μL) was added to adjust the total volume and the tubes were placed in the luminescence reader. KCN, acetonitrile, or benzonitrile solution (50 μL) was injected to initiate chemiluminescence and the cover was closed. The signal was displayed and integrated for a 10 s interval, in arbitrary units. The kinetics of the chemiluminescence reactions were monitored on a recorder connected to the luminescence reader.

CL imaging of isoluminol-labeled streptavidin on the microplate or membrane

Samples were scaled down and pipetted into the wells of the microplate or dotted directly on the membrane as described above. After addition of Na_2CO_3 (0.1 mol L^{-1}, 100 μL), H_2O_2 (0.1 mol L^{-1}, 20 μL), distilled water (30 μL), and acetonitrile (50 μL) into the microplate or immersing the membrane into the CL cocktail solution (2 mL 50 mmol L^{-1} Na_2CO_3, 10 mmol L^{-1} H_2O_2, 25% acetonitrile), the microplate or membrane was placed in a densitometric luminometer. The CL image was detected for 30 s, 2 min, or 5 min exposure.

Antibody CL imaging on the microplate

The wells of the microplate were coated by adding 100 μL of different concentrations of biotinylated IgG in bicarbonate–carbonate buffer (35 mmol L^{-1} $NaHCO_3$, 15 mmol L^{-1} Na_2CO_3, 0.05% NaN_3, 250 μg mL^{-1} bovine serum albumin (BSA), pH 9.0) for at least 15 h at 4 °C. The wells were washed three times with 0.2 mL PBS (10 mmol L^{-1} $Na_2HPO_4.2H_2O$, 1 mmol L^{-1} KH_2PO_4, 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 0.02% NaN_3, pH 7.4) containing 0.05% Tween 20 and blocked for at least 4 h in PBS containing 3% BSA. The wells were then washed once with 0.2 mL PBSTB (PBS containing 0.05% Tween 20 and 1% BSA). A stock solution of isoluminol-labeled streptavidin (1 mg mL^{-1} in water) was diluted with PBSTB and 0.1 mL of the diluted solution (10 μg mL^{-1}) was added to each well. The plate was incubated for 1 h at room temperature on a shaker and then washed three times with 0.2 mL PBSTB and once with water. After adding Na_2CO_3 (0.1 mol L^{-1}, 100 μL), H_2O_2 (0.1 mol L^{-1}, 20 μL), distilled water (30 μL), and acetonitrile (50 μL) to the wells the microplate was placed in a densitometric luminometer. The CL image was detected for 3 min exposure.

CL imaging of labeled DNA on a nylon membrane

Different amounts of biotinylated DNA were spotted directly on a membrane as described above. After heating with an electric drier the membrane was immersed in a coupling solution containing PBS buffer (800 μL), BSA (2 mg mL^{-1}, 200 μL), Tween 20 (1 μL) and isoluminol-labeled streptavidin (1 mg mL^{-1}, 20 μL) at 37 °C for 1 h. The membrane was then washed three times with the same coupling buffer, but without isoluminol-labeled streptavidin, and once with water. The membrane was immersed in the CL cocktail solution, described above, for 5 s, the membrane was then quickly placed in a densitometric luminometer. The CL image was detected for 3 min exposure.

Results and discussion

Luminol is one of the most commonly used chemiluminescence (CL) reagents in aqueous alkaline solution. Luminol is oxidized to 3-aminophthalate with emission of light. The luminol CL reaction system most often used is luminol+H_2O_2+catalyst+OH^-, which has been widely used to determine luminol-type compounds in biochemical analysis. Although many non-enzymatic and sensitive luminol reactions are reported, none is sufficient robust and easy to handle for CL imaging in routine applications. Most of these CL reactions require the rapid addition of oxidant at highly alkaline pH with a catalyst. For example, when the CL reaction with luminol is initiated by use of KCN as a catalyst in the presence of dissolved oxygen addition of H_2O_2 can further increase the CL signal. As shown in Fig. 1a, the kinetics of this reaction are very rapid and it is too difficult to register the maximum light intensity. Even after a few seconds the CL is decaying rapidly and the reaction is over within 20 s. By use of this CL reaction luminol could be detected at levels as low as 500 fmol/tube with a batch method, but this kind of CL reaction is difficult to couple with CL imaging because of the rapid kinetics. A CL reaction of at least a few minutes is generally required for easy handling of CL imaging, because of lag time between initiation of the reaction and data collection. As usual, use of a sub-optimum reaction pH could slow down the reaction, at the expense of low

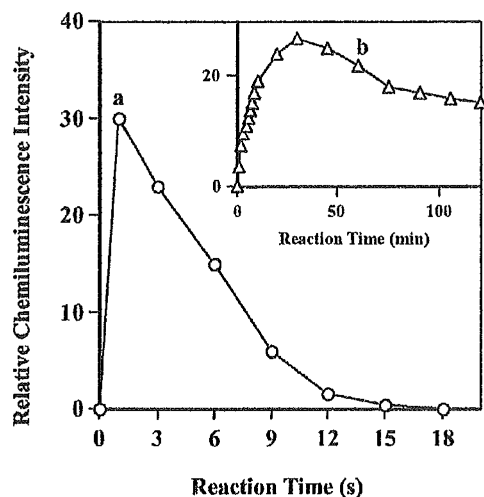

Fig. 1 Time course of the kinetic profile of the CL reaction of 100 pmol/tube luminol in 50 mmol L^{-1} Na_2CO_3 with (*a*) 22 mmol L^{-1} KCN and 1 mmol L^{-1} H_2O_2 and (*b*) 0.12% acetonitrile and 10 mmol L^{-1} H_2O_2

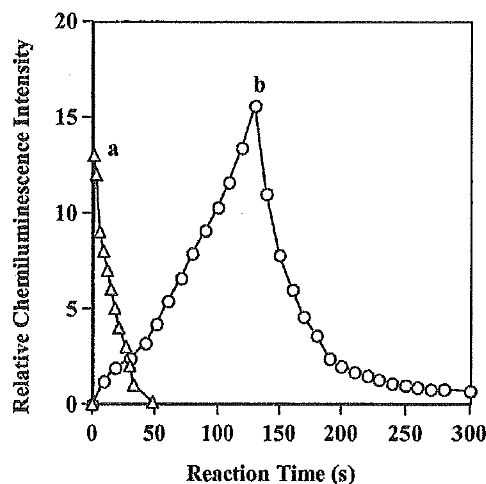

Fig. 2 Time course of the kinetic profile of the CL reaction of 1 nmol/tube fluorescein with (*a*) 22 mmol L^{-1} KCN and (*b*) 10% acetonitrile in the presence of 50 mmol L^{-1} Na_2CO_3 and 20 mmol L^{-1} H_2O_2

sensitivity, but this is not a suitable way of controlling the CL kinetics for practical applications.

As suggested by McKeown et al. [15], singlet oxygen produced by reaction between KCN and H_2O_2 in basic alkaline solution is responsible for luminol CL. Indeed, the addition of a singlet oxygen quencher, e.g. sodium azide, dimethylfuran, or 1,4-diazabicyclo(2,2,2)octane [16] inhibited CL intensity by more than 50%. We therefore postulated that substitution of KCN by other nitrile compounds might slow the rate of formation of singlet oxygen, and the speed of the CL emission would be accordingly reduced. As shown in Fig. 1b, the use of CH_3CN instead of KCN indeed leads to much slower CL emission. The maximum intensity was reached after 30 min and the signal was still greater than 50% of peak height even after 2 h. Importantly this CL intensity could be greatly enhanced by further increasing the concentration of acetonitrile, and the rate of the CL reaction was also increased accordingly. With a 25% acetonitrile in the reaction mixture the maximum CL peak was reached approximately 1.5 min after mixing of the reagents and then the signal slowly decreased. This characteristic is quite important for developing simple and highly reproducible CL imaging detection, because this longer-lived signal, occurring over a period of minutes, simplifies the handling of CL imaging. It was also found that use of benzonitrile could further slow down this CL reaction, probably because the CL reaction occurred by nucleophilic attack on a slightly more electrophilic carbon of the nitrile group and steric hindrance was less in the acetonitrile than in the benzonitrile. These results indicated that the nitrile group played a major role in these CL reactions. The superoxide radical was found in the KCN-containing CL reaction whereas the hydroxyl radical was found in the nitrile-containing CL reactions. This might be because of different mechanisms for KCN and nitriles. The CL spectrum for H_2O_2–acetonitrile–luminol was identical with that for the conventional H_2O_2–horse radish peroxidase–luminol reaction, with a maximum wavelength of approximately 425 nm. The mechanism could therefore be initial formation of oxygen species between H_2O_2 and KCN or nitrile compounds, then reaction with luminol to emit CL.

Similar phenomena were also observed when fluorescein was used instead of luminol, i.e. faster-emission of CL by the H_2O_2–KCN–fluorescein system and a slower emission by H_2O_2–acetonitrile–fluorescein (Fig. 2). Overall, all of these results further supported the above-mentioned mechanism.

Second, the sensitivity was investigated to determine whether the proposed reaction conditions were suitable for tuning of CL kinetics. The measurable range of luminol at a concentration of 25% acetonitrile was 10 pmol to 10 fmol/tube. With our simple arrangement, luminol was detectable at a concentration as low as 1 fmol/tube. This demonstrated sensitivity was comparable with that of the most sensitive current luminol CL methods [17]. On the basis of on these results we believed that by modifying the chemical property of the catalyst the CL kinetics could be favorably controlled for CL imaging, i.e. faster-emitting CL reactions could be tuned to slower-emitting reactions.

We subsequently showed that this CL reaction could be easily used for CL imaging detection of a universal marker, commercially available isoluminol-labeled streptavidin. The biotin–streptavidin complex has been found to be a useful tool in numerous bioanalytical applications [18, 19, 20, 21, 22]. Furthermore, by use of this single isoluminol-labeled streptavidin substance a variety of DNA, antigens, and antibodies, etc., could easily be detected. The measurable range of the conjugated protein was 33 pmol–33 fmol/well, and as little as 16 fmol/well was detectable by use of our CCD camera (Fig. 3). It should be mentioned that the simple CCD camera used was cooled

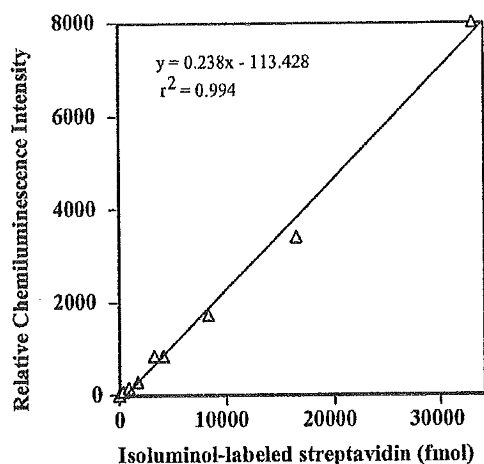

Fig. 3 Calibration curve for isoluminol-labeled strepatavidin obtained by microplate CL imaging with an exposure time of 2 min

to –25 °C. A lower CCD camera operating temperature (–120 °C) of has been reported to improve the sensitivity [23]. According to Sigma, moreover, only 3.5 isoluminol molecules were attached to one streptavidin molecule. Many researchers indicated that increasing the number of labels per protein was associated with an increase in sensitivity [24, 25, 26, 27]. With this CL imaging reaction it can, therefore, be expected that the detection limit of 16 fmol labeled streptavidin/well can be further improved either by use of lower temperature or increasing the labeling of the protein by use of microspheres [25], polymeric backbones [26], or even liposomes [27].

We also showed it was extremely easy to apply the controlled CL reaction to CCD imaging for location of a labeled probe, without the need to increase the viscosity of the CL solution to improve spatial resolution. The procedure was rather simple and economical for general use. By immersing the spotted membrane in CL cocktail solution containing 50 mmol L^{-1} Na_2CO_3, 10 mmol L^{-1} H_2O_2, and 25% acetonitrile for 10 s and then placing the membrane in a densitometric luminometer the CL image could be acquired with a 2-min exposure time. As shown in Fig. 4, the spots of the labeled probes were clearly located on the membrane. The size of the image was related to the volume spotted and the intensity of the image was proportional to the concentration of the labeled probe.

As a preliminary application, this simple method was used for imaging detection of DNA and protein. In recent years there has been considerable interest in the development of DNA and protein methods, because of their numerous applications, e.g. the analysis of unknown or mutant genes, disease diagnosis, etc. It is well known that DNA or protein can be easily absorbed or transferred on to a membrane or microplate where they can then be easily biotinylated by use of many commercial biotin-labeling reagents, such as biotin-succinimide ester and photoprobe biotin, etc. As a first example, we physically adsorbed dif-

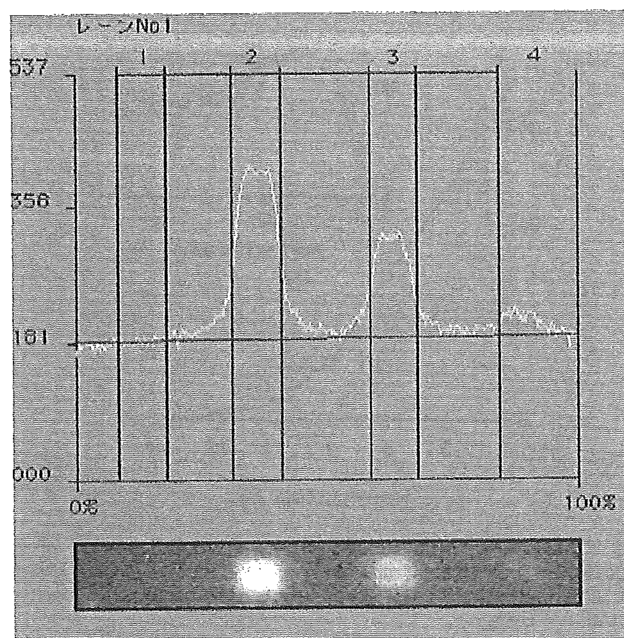

Fig. 5 CL images of biotinylated IgG on the microplate: *1*, blank; *2*, 1000-fold; *3*, 5000-fold; *4*, 10,000-fold dilution

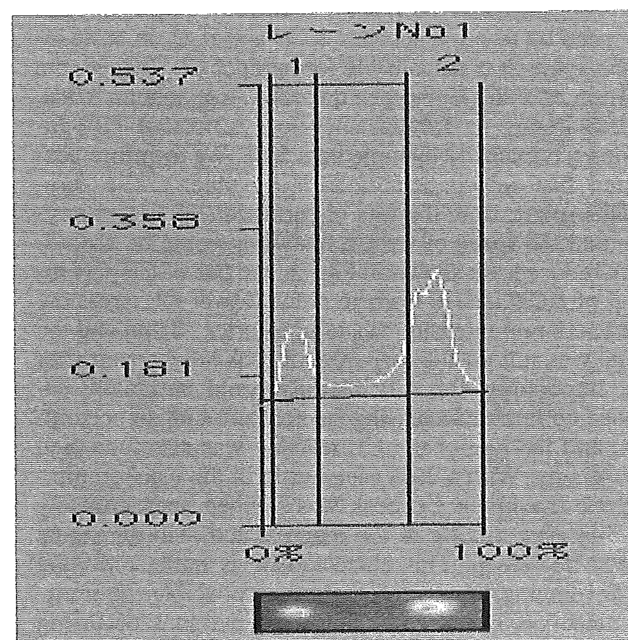

Fig. 6 CL images of nylon membrane with spotted biotinylated DNA: *1*, 400 ng; *2*, 800 ng

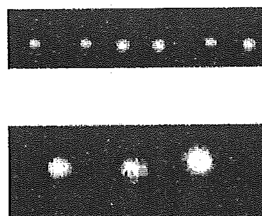

Fig. 4 CL image of nylon membrane with spotted isoluminol-labeled streptavidin: *top*, 800 fmol/0.5 μL; *bottom*, 1.6 pmol/1.0 μL

ferent biotinylated IgG on to the wells of the microplate and then obtained an image simply by use of isoluminol-labeled streptavidin. As shown in Fig.5, biotinylated IgG could be easily visualized even after 10,000-fold dilution.

As a second example, this simple imaging method was further illustrated by detection of DNA by use of the same biotin-strepatavidin chemistry. Biotinylated DNA samples were dotted on the nylon membrane then reacted with isoluminol-labeled streptavidin for 1 h. For this specific DNA sample an amount of DNA between 20 ng and 1 μg was easily visualized on the membrane; a typical image is shown in Fig. 6.

Conclusions

In summary, the speed of CL kinetics could be reduced for simple CL imaging by modifying the chemical properties of the catalyst. By means of the proposed CL reaction high sensitivity could be achieved even with a simple and inexpensive setup. The non-enzymatic luminol CL reaction was used for simple and economical CL imaging of labeled protein in a microplate and location of labeled protein and DNA on a membrane. Overall, the proposed CL reaction might broaden many non-enzymatic CL reactions for different applications. We are currently using the proposed reaction for simple CL imaging of protein and DNA arrays.

Acknowledgement This work was supported by grants from Japan Society for the Promotion of Science.

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Analytica Chimica Acta 381 (1999) 155–163

ANALYTICA CHIMICA ACTA

A chemiluminescence derivatization method for detecting nucleic acids and DNA probes using a trimethoxyphenylglyoxal reagent that recognizes guanine

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Received 16 June 1998; received in revised form 5 October 1998; accepted 11 October 1998

Abstract

A chemiluminescence derivatization method using a novel reagent, 3′,4′,5′-trimethoxyphenylglyoxal (TMPG) was studied for the quantitative detection of nucleic acids and deoxyguanylic acid oligomers on a nylon membrane. In the method, TMPG rapidly converted guanine of a nucleic acid to a chemiluminescent derivative in an alkaline phosphate solution at a room temperature. A cooled, charge-coupled device camera was used to detect the chemiluminescence emitted at room temperature from the derivatives on the membrane after the TMPG reaction. The detection signal was increased by increasing the amount of deoxyguanylic acid in the analyte molecule. When a DNA probe having a 5′-$(G)_{15}TT(G)_{15}TT$ deoxyoligomer at its 3′ terminus was hybridized with a target complementary DNA fragment on the membrane, an increased chemiluminescence from the hybrid was detected by the proposed method. The lower limit of detection is approximately 1.0 pmol of the target DNA fragment bound to the probe in the hybridization assay. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Chemiluminescence; Derivatization; Guanine; Nucleic acid; DNA probe; Trimethoxyphenylglyoxal

1. Introduction

Non-radiochemical methods utilizing a fluorescent or chemiluminescent reagent for detecting DNA probes or primers are very desirable [1]. They allow the development of powerful analytical techniques for obtaining information on gene structure and function. Recent advances include the use of laser-induced fluorescent dyes such as fluorescein analogues, which have been used to replace radioisotope-labelled oligodeoxyribonucleotides in automated DNA sequence analysis [2,3] and hybridization assays [4]. Another recently developed reagent was a chemiluminescent substrate of alkaline phosphatase, 3-(2′-spiroadamanatane)-4-methoxy-4-(3″phosphoryloxy)phenyl-1,2-dioetane (AMPPD) [5–7]. When alkaline phosphatase attached to a DNA probe or primer is allowed to react with AMPPD, a signal amplification technique can detect an increased chemiluminescence depending on the enzymatic reaction time. On the other hand, we

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PII: S0003-2670(98)00724-7

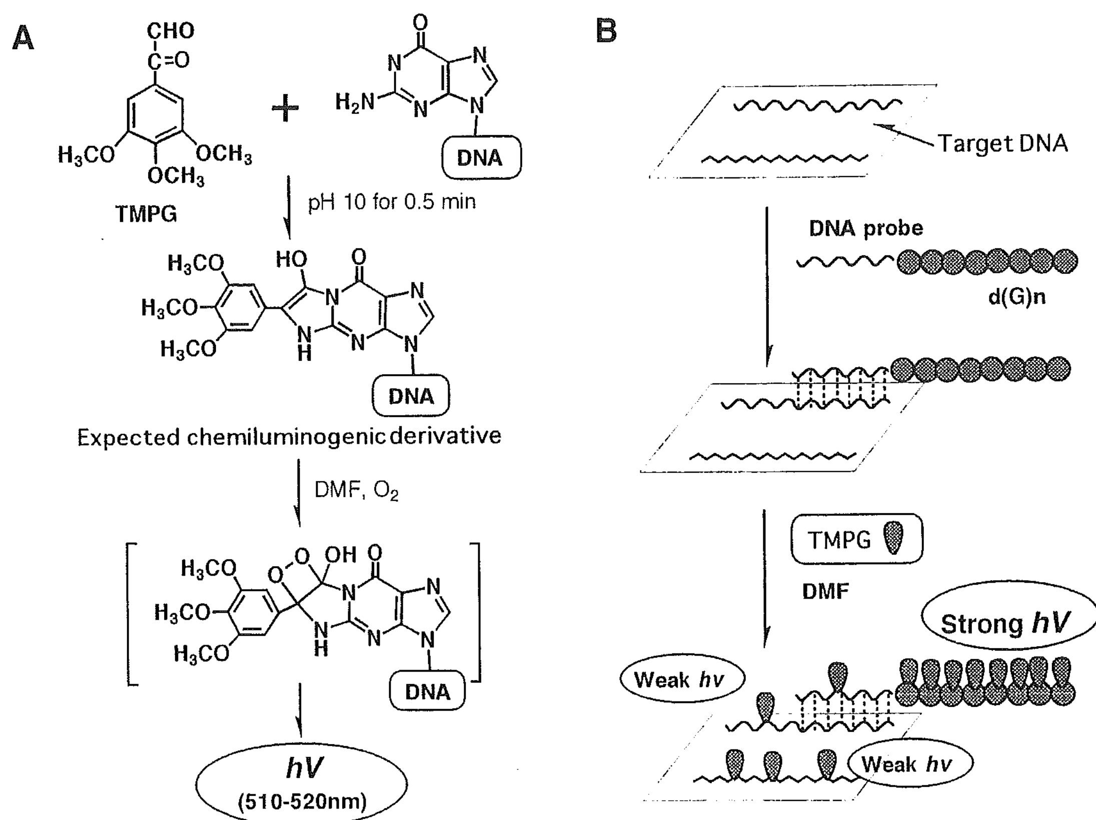

Fig. 1. (A) A possible pathway for the derivatization and chemiluminescence rections of nucleic acids with TMPG. The chemical structure of the product was considered on the basis of a reaction product between methylglyoxal and guanine reported by Shapiro et al. [12]. (B) Schematic illustration of chemiluminescence detection in a hybridization assay of a DNA fragment bound to a probe with a $d(G)_n$ tail. *hν* is the chemiluminescence light emitted by the products of the reaction between guanine and TMPG.

have recently found that phenylglyoxal (PGO) reacts with guanine nucleotides to produce fluorescent derivatives [8,9], which emit chemiluminescence in a weakly alkaline solution [10].

In this paper, we found that 3′,4′,5′-trimethoxyphenylglyoxal (TMPG), one of PGO analogues, was more useful for the chemiluminescence detection of guanine bases in nucleic acids. We then studied a chemiluminescene derivatization method (Fig. 1(A)) for detecting DNA and polydeoxyguanylic acids ($d(G)_n$) on a blotting membrane. The chemiluminescene detection was performed by a densitometric luminometer equipped with a cooled, charge-coupled device (CCD) camera. This method was further applied to the detection of a hybrid with a DNA probe having a $d(G)_n$ tail in a hybridization system (Fig. 1(B)). The sensitivity of the proposed method should be increased by guanine-repeated amplification.

2. Experimental

2.1. Materials

The nucleic acid bases, nucleocides, nucleotides and salmon testes DNA were obtained from Seikagaku-Kogyo (Tokyo, Japan) and Sigma (St. Louis, MO, USA). TMPG and its analogues were synthesized using a previously described method for tryptophan analysis [11]. Phenylglyoxal monohydrate was purchased from Sigma–Aldrich Japan (Tokyo, Japan). The deoxyguanylic acid oligomers, DNA fragments and probes were synthetic products from Toowa–Gohsei (Tokyo, Japan). Other reagents were of the highest purity available. Nylon membranes (Clear Blot Membrane-*N* AE-6650) were obtained from Atto (Tokyo, Japan).

2.2. Solution reaction and detection with a photon-counting luminometer

A portion (100 µl) of an analyte dissolved in H_2O was mixed with 100 µl each of 50 mM sodium phosphate buffer (pH 6.5) and 0.2 M TMPG dissolved in dimethyl sulfoxide (DMSO). Water, instead of the sample, was used for a blank. The mixture was then warmed at 37°C for 20 min to produce the derivatives. In succession, 20 µl of 0.2 M sodium phosphate (pH 8.0), 20 µl of 0.25 M H_2O_2 and 160 µl of dimethylformamide (DMF) were added to 40 µl of the derivative mixture. After mixing for about 1 s, the chemiluminescence intensity in the mixture was measured at ambient temperature (26°C) for 0.5 min by a photon-counting computer-controlled Lumat LB-9051 luminometer (Berthold Japan, Tokyo, Japan), using 75×12 mm round-bottom glass tubes.

2.3. Membrane reaction and detection with a CCD camera

Each sample in H_2O (2 µl) was dotted on a nylon membrane. The spots were dried with an electric drier for a few minutes. The membrane was moistened with 0.2 M sodium phosphate aqueous solution (pH 10) for a few seconds, and then immersed in a 0.3 M TMPG solution for 0.5 min at ambient temperature (26°C). The moist membrane was then dipped in DMF for a few seconds, and quickly placed in a densitometric luminometer. The chemiluminescence image was detected in a 0.5 min exposure at ambient temperature (26°C) by a Lumino CCD AE-6930 densitograph (Atto, Tokyo, Japan) and processed in a computer using Densitometer Analyst version 4.0 software.

2.4. Hybridization with a DNA probe on a membrane

DNA fragments and a $d(G)_{30}$ probe (10 pmol each per 2 µl) were spotted on a nylon membrane. After baking with an electric drier, the membrane was immersed in a hybridization buffer consisting of 60 mM sodium citrate buffer (pH 7.0) and 0.6 M NaCl in the presence or absence of 50 nM of the probe at 42°C for 2 h. The membrane was then washed in a mixture of 0.1% sodium dodecyl sulfate, 30 mM sodium citrate buffer (pH 7.0) and 0.3 M NaCl for 1 min. Then the membrane was washed with H_2O for 1 min. The membrane was placed in the TMPG solution, and the chemiluminescence images were detected with a CCD camera in the same manner as described above.

3. Results and discussion

3.1. Conditions for the reaction with TMPG

We first studied the reactivities of the PGO analogues such as 4′-methoxyphenylglyoxal (MPG), 3′,4′-dimethoxyphenylglyoxal (DMPG), TMPG and 3′,4′-methylenedioxyphenylglyoxal (MDPG) in order to find a promising chemiluminogenic reagent for a nucleic acid. These arylglyoxals as well as PGO produced chemiluminescence with guanine under neutral reaction conditions, but not with the other nucleic acid bases and nucleotides (e.g., adenine, cytosine, uracil, thymine, adenosine-5′-monophosphate (AMP), cytidine-5′-monophosphate (CMP)). Of these reagents, TMPG produced the most intense chemiluminescence for salmon testes DNA (Fig. 2). The chemiluminescent derivative was produced by the reaction at 37°C with TMPG in sodium phosphate buffer of pH 6.5 for 20 min or at pH 7.5 for 2 min under almost the same conditions as those [10] described for the PGO reaction.

The derivative in the reaction mixture emitted chemiluminescence more intensely by oxidation with H_2O_2 in the presence of DMF (Fig. 3(A)). The maximum number of photons was counted 10 s after the addition of DMF and H_2O_2. Generally, chemiluminescence occurs in dioxetane formation of the luminogenic molecule by oxidation [7]. In this study, we found that the resulting chemiluminogenic derivative of DNA bound to a nylon membrane emits a strong chemiluminescence in DMF without H_2O_2 (Fig. 3(B)). The results suggest that the derivative bound to the membrane is more liable to oxidation. Therefore, we examined the reaction conditions for producing the derivative of TMPG with the membrane-bound samples of salmon testes DNA and a deoxyguanylic acid 20 oligomer ($d(G)_{20}$).

The nucleic acids on the membrane reacted rapidly (30–60 s) with TMPG at pH 9–10, as shown in Fig. 4. Consequently, we developed a quick and easy procedure described in Section 2 to detect the luminescence

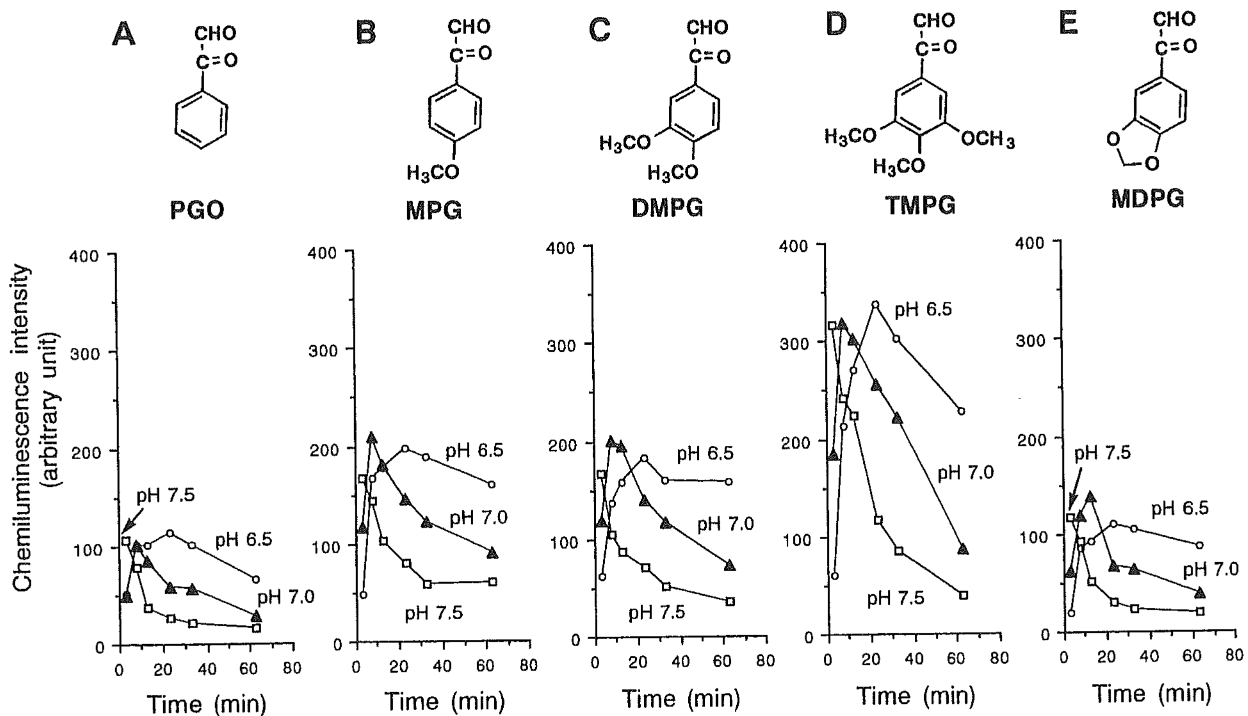

Fig. 2. Effects of the reaction time and the pH of the sodium phosphate buffer, on the production of luminescent DNA derivatives by arylglyoxals in the reaction mixtures. Arylglyoxals: (A) PGO, (B) MPG, (C) DMPG, (D) TMPG, and (E) MDPG. A portion (100 µl) of 30 µg/ml salmon testes DNA in a glass tube was treated according to the procedure recommended for the solution reaction, except that the PGO analogues and 50 mM sodium phosphate buffers at pH 6.5–7.5 were used for various reaction times.

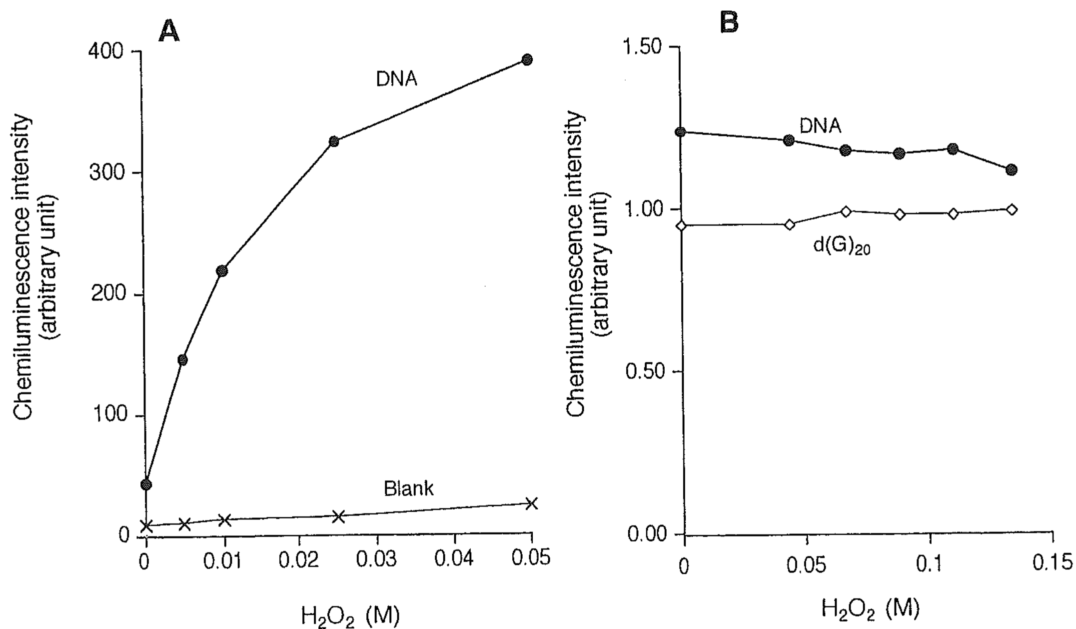

Fig. 3. Effect of H_2O_2 concentration in the oxidation mixture on the chemiluminescence development from the TMPG derivatives of (A) DNA and its blank in the reaction mixture, and of (B) DNA and $d(G)_{20}$ on a nylon membrane. For the experiment of (A), a portion (100 µl) of 30 µg/ml salmon testes DNA or water was treated according to the procedure recommended for the solution reaction, except that 0.05–0.5 M (final conc., 0.005–0.05 M) H_2O_2 aqueous solutions were used. For the experiment of (B), 2 µl each of 0.3 µg/µl DNA and 0.06 µg/µl $d(G)_{20}$ was treated according to the procedure recommended for the membrane reaction, except that DMF solutions containing 10% of 0.5–1.5 M (final conc., 0.05–0.15 M) H_2O_2 were used.

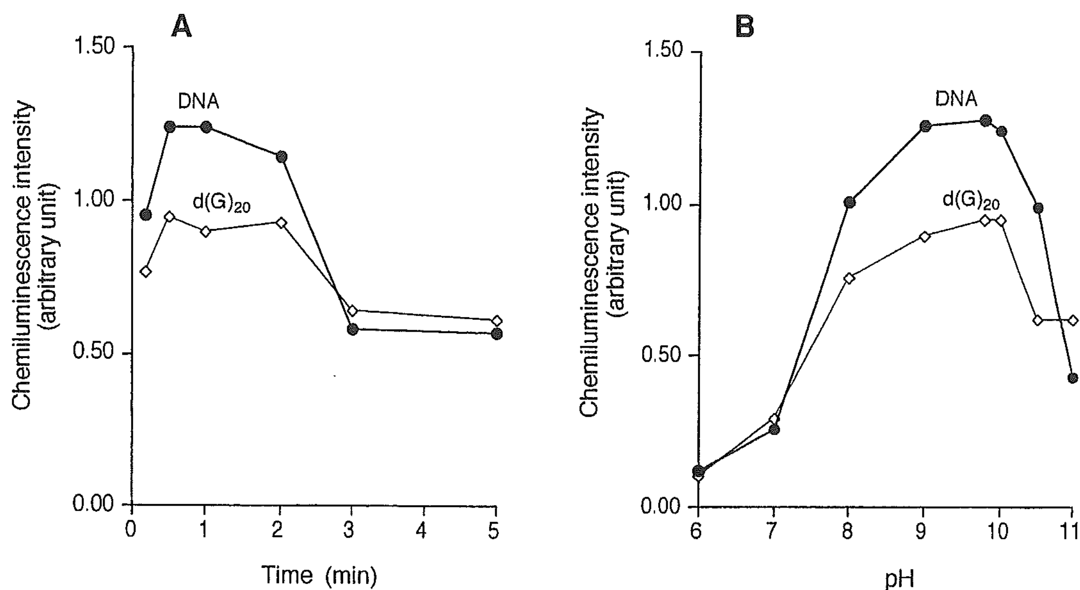

Fig. 4. Effect of (A) the reaction time and (B) the pH of the sodium phosphate solution, on the production of the TMPG derivatives of DNA and $d(G)_{20}$ on a nylon membrane. The samples (2 μl each) of 0.3 μg/μl DNA and 0.06 μg/μl $d(G)_{20}$ were treated according to the procedure recommended for the membrane reaction, except that the reaction time and the pH of 0.2 M sodium phosphate (composition, NaH_2PO_4–Na_2HPO_4–Na_3PO_4) were varied.

of the TMPG derivatives on the membrane, using a densitometric luminometer equipped with a cooled CCD camera. Each sample on the membrane can be processed in about 2 min. This includes 0.5 min for the reaction with TMPG at pH 10 and the 0.5 min exposure time for the detection.

It was reported that aliphatic glyoxals such as methylglyoxal and glyoxal react with guanine base [12]. However, they did not make guanine or DNA luminescent under neutral or alkaline reaction conditions. Recently, PGO and the alkylglyoxals were found to react with adenine base under strongly acidic conditions when heated at 100°C, and to then produce chemiluminescence under alkaline conditions [13,14]. Under the drastic reaction conditions, however, guanine or its containing nucleotides could not be made luminescent by the glyoxals.

3.2. *Chemiluminescence signals detected by CCD camera*

A method of detecting chemiluminescence with a cooled (−120°C) CCD camera attached to a computer digital evaluation system has recently been adapted for low light imaging of the chemiluminescence, which was emitted from a hybrid of a plasmid DNA (2.5×10^3 bases) as low as sub-attomole (2.5 pg) by an alkaline phosphatase-conjugated probe and AMPPD system [15]. We used a similar CCD camera, but it was cooled at −25°C.

Fig. 5(A) shows the chemiluminescence images and intensities from the reaction mixtures of salmon testes DNA and synthetic oligomers of $d(G)_{20}$, $d(G)_{15}$ and $d(G)_{10}$ with TMPG in the wells of a microtiter plate. The CCD camera could detect the chemiluminescent signal from 300 ng of the DNA in the reaction mixture, which was twice as strong as the background noise. However, a photon-counting luminometer used for Figs. 2 and 3(A) can detect the same reaction mixtures 100-fold more sensitively. Thus, the sensitivity of the used CCD camera is relatively low, though its sensitivity may be improved by cooling the CCD part to increase the signal-to-noise ratio.

The CCD camera at −25°C was used for the detection of the chemiluminescences emitted from the samples on a nylon membrane after the TMPG reaction. The detection signal (Fig. 5(B)) for the membrane-bound samples was nearly 80 times greater than that (Fig. 5(A)) for the microtiter samples. In this case, the detector response (Fig. 5(A)) for the latter samples was gained 50 times, so that the background intensity for the titerplate was higher than that for the

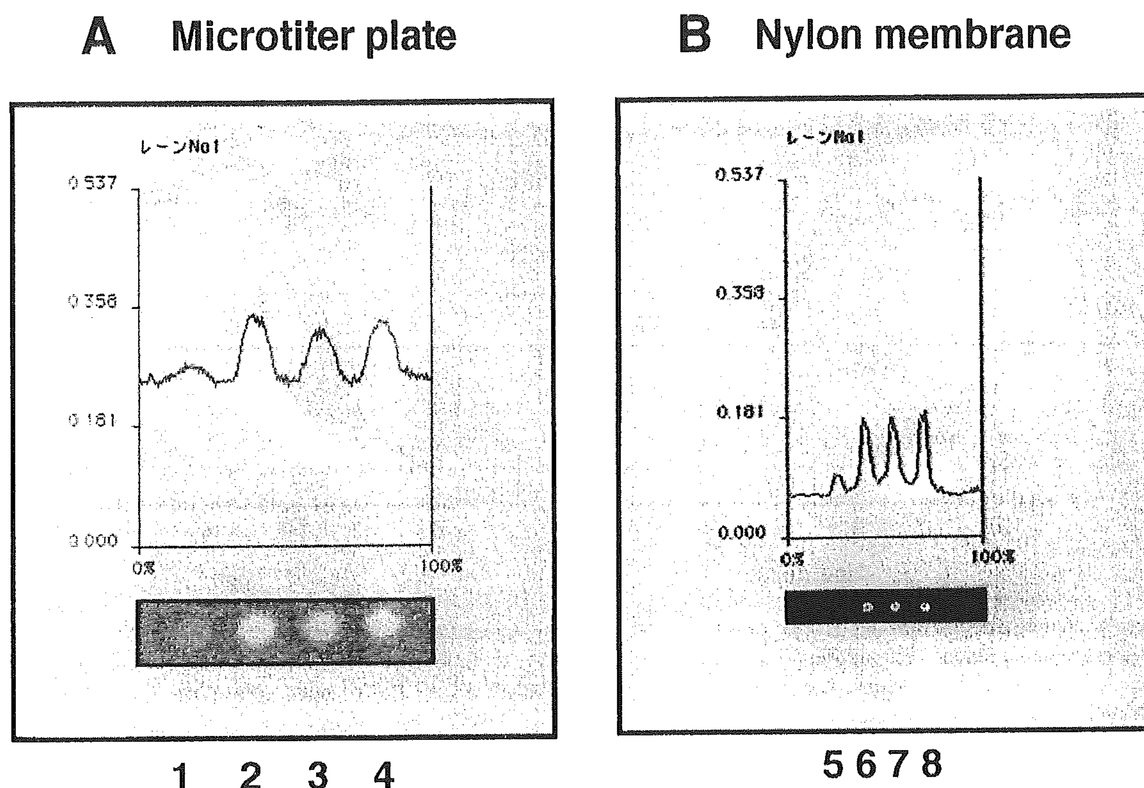

Fig. 5. Detection of the chemiluminescent TMPG derivatives of DNA, $d(G)_{20}$, $d(G)_{15}$ and $d(G)_{10}$ in (A) the wells of a microtiter plate, or on (B) a nylon membrane, by a cooled CCD camera. Samples [ng per well or spot]: (1) salmon testes DNA [670], (2) $d(G)_{20}$ [830], (3) $d(G)_{15}$ [810], (4) $d(G)_{10}$ [750], (5) salmon testes DNA [100], (6) $d(G)_{20}$ [120], (7) $d(G)_{15}$ [120], and (8) $d(G)_{10}$ [110]. The samples in the wells or on the membrane were tested according to the procedures recommended for the solution and membrane reactions, respectively, and their produced chemiluminescences were detected by the CCD camera.

membrane. For the same mass concentration (700–800 ng per well or 100–120 ng per spot) of the analytes, the chemiluminescence produce by $d(G)_{10-20}$ was almost as intense, and approximately 4 times greater than that of salmon testes DNA (Fig. 5), because the guanine content in the DNA molecule is probably one fourth. The detection limits at a signal-to-noise ratio of 2 on the membrane were approximately 1 ng for the $d(G)_{10-20}$ (molecular weight, 3230–6522) and 4 ng for the DNA (genome size, about 3×10^9 base-pair molecular weight, about 2×10^{12}). These mass amounts correspond to 0.15–0.3 pmol for the $d(G)_{10-20}$ and approximately 2 zmol for the DNA as a genome. Therefore, the presented method gives a high sensitivity for the nucleic acid containing a large amount of guanine base in the molecule.

Quantitative measurements were performed on the membrane using salmon testes DNA and $d(G)_{10-20}$. The calibration graph for each sample, as shown in Fig. 6, is linear with a correlation coefficient (r) between 0.9867 and 0.9959. The relative standard deviation (RSD) of the chemiluminescence intensity, based on the integral area, was 7.8% when 0.5 µg DNA (n=15) was repeatedly reacted with TMPG. At an equimolar concentration of 4–16 pmol, the $d(G)_{20}$ oligomer produced twice the signal intensity of the $d(G)_{10}$ oligomer (Fig. 6(B)). Therefore, the detection signal for $d(G)_n$ can be increased by increasing the guanine content of the nucleic acid molecule.

Fig. 7 shows the comparing of the data of the calibration graph of DNA (0–0.2 µg) obtained by the present chemiluminescence method and a popular fluorescent method with ethidium bromide (EBr). The EBr reagent is an intercalator that binds to double-stranded DNA, and gives an increased fluorescence intensity by increasing the concentration of DNA. Nevertheless, we used two different concentrations of EBr for the determination of DNA, a good linearity of the graph was not obtained, and the blank fluores-

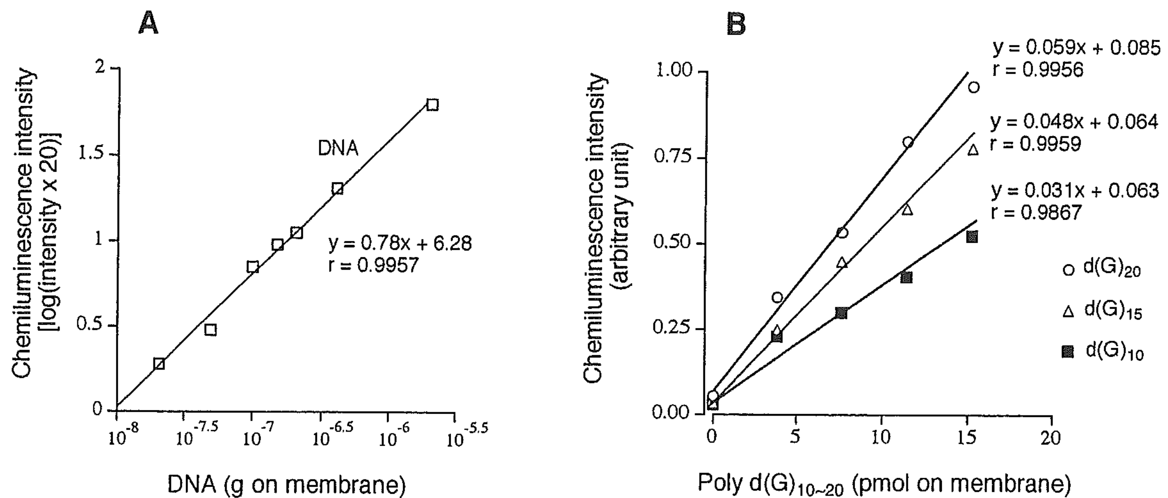

Fig. 6. Calibration graphs for (A) salmon testes DNA and (B) deoxyguanylic acid oligomers $d(G)_{20}$, $d(G)_{15}$ and $d(G)_{10}$ on a nylon membrane. The samples were treated in the same way as described in Fig. 5(B).

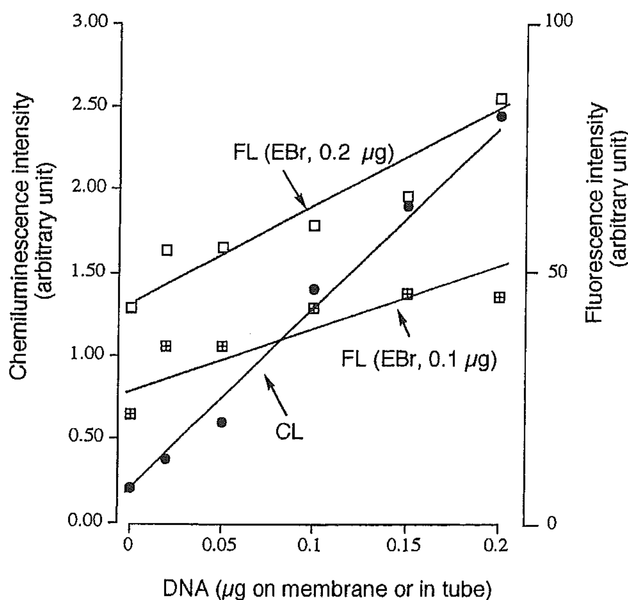

Fig. 7. Calibration graphs of DNA obtained by the present chemiluminescence (CL) method and a fluorescence (FL) method using ethidium bromide (EBr) as an intercalator. For the FL determination, a portion (100 μl) of 0.2–2.0 μg/ml salmon testes DNA was mixed with 100 μl of 1 or 2 μg/ml EBr in 0.2 M NaCl and 0.2 M Tris–HCl buffer (pH 7.5) (1:1, v/v). The fluorescence intensity was measured manually by a Hitachi F-2000 spectrofluorimeter at 480 nm of excitation wavelength and 600 nm of emission wavelength.

cence intensity was relatively high. In comparison, the present method offers more sensitive and reliable signals than the EBr method.

3.3. *Detecting the chemiluminescence of hybrid DNA bound to a membrane*

The preceding results led to the design of a new hybridization-assay protocol using a cDNA probe with an attached $d(G)_n$ oligomer to increase its chemiluminescence, as illustrated in Fig. 1(B). In this study, a DNA probe with a $d(G)_{30}$ tail covalently attached to its 3′-terminus (5′-CACAATTCCACA-CAAC-$(G)_{15}$TT$(G)_{15}$TT-3′), its complementary DNA target (5′-CTCGTATGTTGTGTGGAATTGTGAGC-GGAT-3′) and a non-target DNA fragment (5′-TCG-TGACTGGGAAAACCCTGGCGTTACCCA-3′) were used to test a fundamental hybridization system.

In the example shown in Fig. 8, the chemiluminescence intensity produced by a hybrid (sample A-2) of the target DNA fragment bound to the $d(G)_{30}$ probe was greater than that of the target DNA alone (sample B-2). This method detected 10 pmol of the target DNA fragement, spotted on a nylon membrane after hybridization with 50 nM of the $d(G)_{30}$ probe, by its intense chemiluminescence. Background chemiluminescence caused by non-specific binding of the probe in the hybridization buffer to the membrane was negligible. In this experiment, the $d(G)_{30}$ probe was also dotted as a reference on the membrane (samples A-1 and B-1) to compare with the non-hybridization image, because the DNA fragments as well as the probe bound to the membrane-bound probe was detected by 0.1 pmol. Therefore, the probe bound to the target DNA frag-

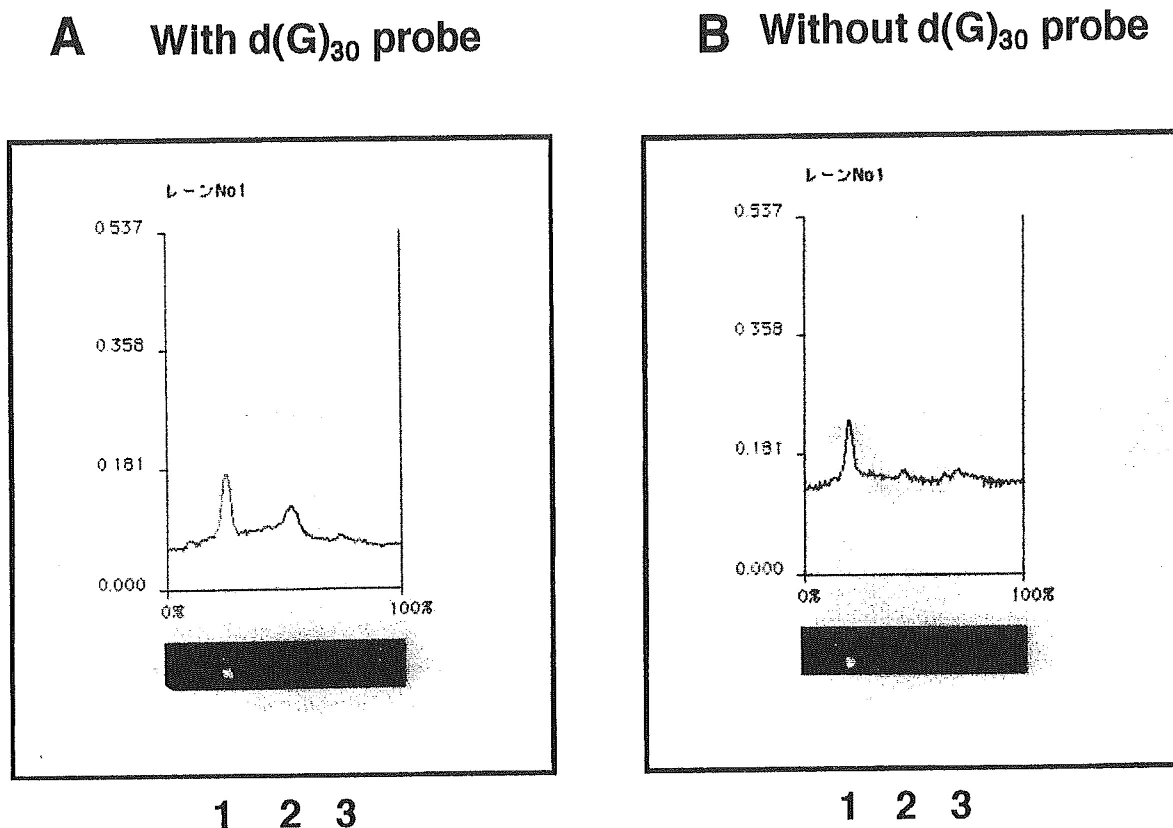

Fig. 8. Hybridization assay of a DNA fragment (30 mer) on a nylon membrane: (A) with a DNA probe with a $(G)_{15}TT(G)_{15}TT$ tail, (B) without the probe. Samples (10 pmol each): (1) probe, (2) target DNA fragment, and (3) non-target DNA fragment. The procedures are described in Section 2.

ment gave a positive and reproducible signal, with a lower detection limit of approximately 1.0 pmol of the target DNA.

In this assay system, a certain amount of the probe hybridized with the target might be removed while washed the membrane after the hybridization reaction, and/or the binding capacity of the probe to the target might be low. With the aim of increasing the detection signal of the hybrid versus its concentration, we will study the effects of much larger amount of $d(G)_n$ in the probe, its binding capacity to the target, and some compounds that enhance chemiluminescence on this assay system.

4. Conclusions

We developed a specific chemiluminescence derivatization (SCD) method for detecting DNA and $d(G)_n$ on a nylon membrane, based on the chemical reaction of guanine moieties of the nucleic acid with TMPG. The SCD method has the advantages of speed and simplicity for the quantitative determination of nucleic acids and DNA probes containing $d(G)_n$. Using the probe with a $d(G)_n$ tail will increase the chemiluminescence in proportion to the amount of guanylic acid in the probe molecule. The SCD technique with TMPG described here may be useful for the in situ detection of guanine-rich regions of the genome. Such regions include telomeres, which consist of hundreds or thousands of tandem repeats of the sequence – TTAGGG – at the end of chromosomes [16].

Acknowledgements

This work was supported by a Grant-in-Aid of Scientific Research from the Ministry of Education, Science and Culture, Japan.