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# Photographic Detection of Fluorescent-Labelled Oligodeoxynucleotide in the Blotting Format by Peroxyoxalate Chemiluminescence

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The preparation of a fluorescent labelled oligonucleotide and its photographic detection by peroxyoxalate chemiluminescence (PO-CL) are described. Fluorescent labelling of an oligonucleotide (15-mer) was performed with naphthalene-2,3-dicarboxaldehyde to give an *N*-substituted 1-cyanobenz[*f*]isoindole (CBI) derivative (CBI-15-mer). For the photographic detection of CBI-15-mer, the bis(2,6-difluorophenyl) oxalate (DFPO)-dimethyl phthalate (DMP) system was selected to obtain a long-lived CL emission. After optimizing the conditions for the CL reaction, the system was applied to the photographic detection, and as little as 250 fmol per spot of CBI-15-mer on a membrane were detected as a visible spot with an instant photographic film. © 1998 John Wiley & Sons, Ltd.

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## INTRODUCTION

A photographic detection technique for a non-isotopic chemiluminescence (CL)-based assay has an increasing promise for the detection and quantification of biologically important compounds because of its high sensitivity, simplicity and ease of handling. This technique does not require specialized or expensive equipment, and thus is convenient in protein blotting and nucleic acid hybridization assays. For this purpose, enzymatically catalysed CL reaction systems using peroxidase-luminol (1) and alkaline phosphatase-adamantyl dioxetanes (2,3,4,5) have been exclusively employed. In general,

non-enzymatically-produced CL, such as that from acridinium esters as labels, decays rapidly (6) and this poses a problem for photographic CL detection.

In this paper we evaluate the scope and limitations of the analytical performance of peroxyoxalate chemiluminescence (PO-CL) for photographic detection of an oligodeoxynucleotide labelled with naphthalene-2,3-dicarboxaldehyde (NDA) on a membrane in a blotting format. NDA is known to react with primary amines to give highly fluorescent *N*-substituted 1-cyanobenz[*f*]isoindole (CBI) derivatives (7–9). A PO-CL detection of CBI derivatives has also been studied and it was reported that primary amines labelled with NDA gave higher sensitivities than those with dansyl chloride and 4-fluoro-7-nitrobenzoxadiazole in combination with bis(2,4,6-trichlorophenyl) oxalate (TCPO) (10).

In this study, several oxalates were evaluated with

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a view to obtaining a long-lived CL emission, and the bis(2,6-difluorophenyl) oxalate (DFPO)-dimethyl phthalate (DMP) system was selected. After optimizing the conditions for CL reaction, the system was applied to the photographic detection of CBI-oligonucleotide on a membrane.

## MATERIALS AND METHODS

### Apparatus

The HPLC system consisted of two pumps (LC-9A) with a SCL-6B system controller (Shimadzu, Kyoto, Japan), a 7125 injector with a 100- $\mu$ L loop (Rheodyne, Cotati, CA, USA), an ODS column (Daisopak SP-120-5-ODS, 250  $\times$  4.6 mm i.d., Daiso, Osaka, Japan), a Shimadzu SPD-9A UV-Vis detector (set at 260 nm) and an RF-500 spectrofluorometer (set at 480 nm with an excitation at 285 nm). HPLC was performed at ambient temperature using a mixture of acetonitrile and 0.1 mol/L triethylammonium-acetate buffer (pH 7.0) as a mobile phase with a gradient elution.

A TD-4000 lumiphotometer (Laboscience, Tokyo, Japan) with a small glass test-tube (50  $\times$  5 mm, i.d.) was used for the CL measurements. A camera luminometer (Camlight 501) was purchased from Analytical Luminescence Laboratory (San Diego, CA, USA) and the instant film used was Polaroid 667 (black and white).

### Reagents

Cyclohexylamine (CHA) and triethylamine (TEA) were obtained from Wako Pure Chemicals (Osaka, Japan) and were used after distillation. Sephadex G-25 and NDA were purchased from Sigma-Aldrich Japan K.K. (Tokyo). DMP, TCPO, DFPO, bis(2,4-dinitrophenyl) oxalate (DNPO), bis[2-(3,6,9-trioxadecyloxycarbonyl)-4-nitrophenyl] oxalate (TDPO) from Wako were used as received. Bis(pentafluorophenyl) oxalate (PFPO) and bis[2,4,5-trichloro-6-(pentyloxycarbonylphenyl)] oxalate (TPPO) were obtained from Tokyo Kasei Kogyo (Tokyo). 4,4'-Oxalyl-bis[(trifluoromethylsulphonyl)imino]trimethylene-bis(4-methylmorpholinium)trifluoromethane sulphate (MPTQ) was synthesized according to the published method (11). All other chemicals and solvents were of analytical reagent grade. Deionized and distilled water was used throughout.

Membranes used were as follows; Tropilon-Plus

from Tropix (Bedford, MS, USA), Hybond-N+ from Amersham (Arlington, IL, USA) and Zeta-Probe from Bio-Rad (Richmond, CA, USA).

The synthesis of an oligodeoxynucleotide [15-mer: d(TCCAGTCACGACGT)] was performed by using a standard phosphoramidite chemistry (12). Incorporation of an alkylamine linker-arm to 5'-terminus of the oligonucleotide was performed during the oligonucleotide synthesis and 6-(trifluoroacetyl-amino)hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite (Glen Research, VA, USA) was employed as a reagent. Molar absorptivity of the oligonucleotide was calculated using the following equation (13):

$$\text{Oligomer Concentration (pmol}/\mu\text{l)} = 100 \times \frac{A_{260}}{(1.54 \text{ nA} + 0.75 \text{ nC} + 1.17 \text{ nG} + 0.92 \text{ nT})}$$

where  $A_{260}$  = stock solution's absorbance at 260 nm; nx = number of residues of base X in the oligonucleotide.

The approximate concentration of a CBI derivative of oligonucleotide (CBI-15-mer) was calculated by using the molar absorptivity of 15-mer at 260 nm plus 50000 which was tentatively deduced from that for 1-cyano-2-N-propyl benz[*f*] isoindole at 252 nm (9).

### Preparation of CBI derivative of CHA and 15-mer

Preparation of a CBI derivative of CHA (CBI-CHA) was performed according to a previously described procedure (8). The crude products were recrystallized from methanol to give reddish-yellow needles; yield, 60%, m.p. 152–154°C. Analysis calculated for  $C_{19}H_{18}N_2$ : C, 83.17; H, 6.62; N, 10.22 (%). Found: C, 82.80; H, 7.01; N, 10.00 (%).

For the preparation of CBI-15-mer, mixture of an aqueous solution of 15-mer (0.5  $\mu$ mol, 300  $\mu$ L), a 0.01 mol/L sodium borate buffer (pH 9.1, 1.2 mL) and a 0.05 mol/L aqueous solution of sodium cyanide (300  $\mu$ L) was placed in a screw-capped vial. To this was added 1.2 mL of 2.5 mmol/L NDA in *N,N*-dimethylformamide (DMF) immediately, and the resultant solution was kept at room temperature for 10 min in the dark. The reaction was monitored by the HPLC as described above, in which CBI-15-mer ( $t_R$ : 29.4 min) was clearly separated from 15-mer ( $t_R$ : 8.8 min); 95.3% of 15-mer was converted to CBI-15-mer within 10 min. The reaction mixture was then applied to a Sephadex G-25 column (20 mL) and developed with 0.1 mol/L triethylammonium-acetate

buffer (pH 7.0) to desalt and to remove the excess NDA. The fractions containing CBI-15-mer were collected and lyophilized. The crude recovery of CBI-15-mer at this stage was approximately 57%. A final purification was performed by the HPLC when necessary. The fluorescence excitation and emission spectra of CBI-15-mer were identical to those for CBI-CHA, but its intensity at 487 nm was about 40% of that for CBI-CHA.

### Procedure for measurement of CL

The CL reaction conditions were optimized with a lumiphotometer as follows: to 150  $\mu$ L of DMP solution of CBI-CHA in a test tube, 50  $\mu$ L each of hydrogen peroxide in *t*-butyl alcohol (*t*-BuOH) and TEA in *t*-BuOH were added. After mixing, 150  $\mu$ L of oxalate in DMP was added and the CL was measured for 30 min with the lumiphotometer. The total CL intensity was defined as the area under the CL decay curve. All experiments were performed at ambient temperature.

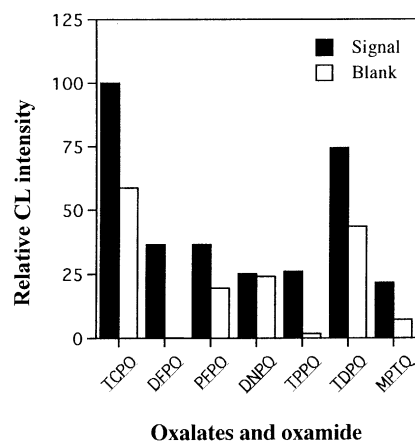
### Photographic detection of CL

The CL measurement by a camera luminometer was performed as follows: 1  $\mu$ L of an aqueous solution of CBI-15-mer was spotted onto a membrane which was pre-wetted with water. After drying completely in the dark, the membrane was dipped in the CL reaction solution consisting of 750  $\mu$ L of 2.0 mmol/L DFPO in DMP, 125  $\mu$ L of 40 mmol/L hydrogen peroxide in *t*-BuOH and 125  $\mu$ L of  $2.0 \times 10^{-5}$  mol/L TEA in *t*-BuOH. The membrane was then taken out of the solution and placed quickly on Saranwrap in the sample compartment of the camera luminometer. The CL produced was imaged on a Polaroid film at room temperature (40 min exposure).

## RESULTS AND DISCUSSION

### Optimization of CL reaction conditions

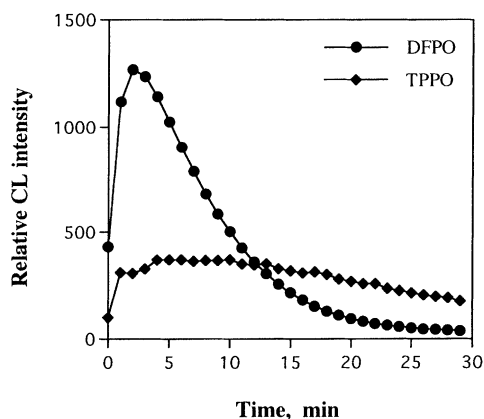
The CL reaction was optimized using CBI-CHA as a standard sample. At first, TCPO, DFPO, PFPO, DNPO, TPPO, TDPO and MPTQ were evaluated as chemiluminogenic oxalates or oxamide to obtain an intense and stable CL emission. As shown in Fig. 1, TCPO and TDPO gave more intense CL but with relatively large blank signals. The CL intensities for



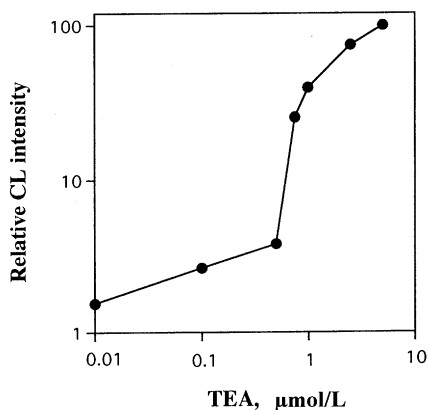
**Figure 1.** Effect of oxalates and oxamide on CL intensity. The concentration of each compound in the CL reaction mixture was: CBI-CHA, 4.7 nmol/L; oxalate or oxamide, 1.5 mmol/L; hydrogen peroxide, 5.0 mmol/L; TEA, 2.5  $\mu$ mol/L; *t*-BuOH, 25%

DFPO and TPPO were less but these showed a higher signal:blank (S:B) ratio. The duration of CL emission was also investigated. The CL emission for TCPO, PFPO, DNPO, TDPO and MPTQ reached a maximum within 80 s after initiation, and then decayed rapidly. By contrast, the emission was slower for DFPO and TPPO and it decayed moderately (Fig. 2); this was considered to be favourable for photographic detection and DFPO, showing more intense CL with the lower blank signal, was selected for further investigations.

The concentration of TEA as a catalyst affected the kinetics and intensity of CL emission. When the



**Figure 2.** Kinetics of CL emission for oxalates. The experimental conditions were same as described in Fig. 1



**Figure 3.** Effect of TEA concentration on CL intensity. The concentration of each compound in the CL reaction mixture was: CBI-CHA, 4.7 nmol/L; DFPO, 3.0 mmol/L; hydrogen peroxide, 5 mmol/L; *t*-BuOH, 25%

concentration of TEA was less than  $5.0 \times 10^{-7}$  mol/L, very weak CL intensities with the delayed peak of CL emission were observed (Fig. 3). As the TEA concentration increased, the time to reach maximum CL intensity decreased slightly in the concentration range  $7.5 \times 10^{-7}$  to  $5 \times 10^{-6}$  mol/L.

The effect of the concentration of hydrogen peroxide on the CL intensity was examined in the range of 1–10 mmol/L (Fig. 4a). The CL intensities increased and the maximum CL emission peaks appeared earlier with an increase in the concentration of hydrogen peroxide. As shown in Fig. 4b, the DFPO concentration also affected the CL intensity; the most

intense CL was observed at 1.5 mmol/L. However, the variation in the concentration of DFPO had almost no influence on the kinetics of CL emission.

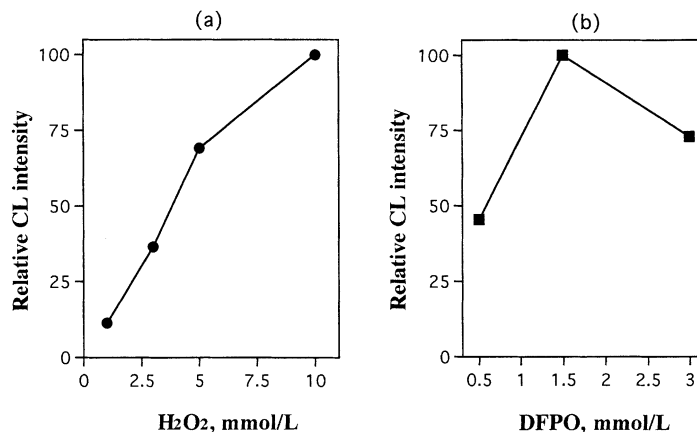
We also studied *t*-BuOH as a diluent for the hydrogen peroxide and TEA. The presence of *t*-BuOH at more than 50% in the reaction mixture drastically reduced the CL intensity; 25% was chosen for further experiments.

### Photographic detection of CBI-15-mer on the membrane

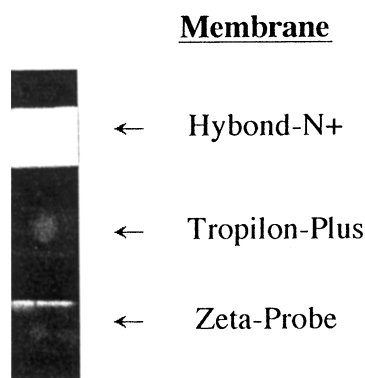
For the photographic detection of CBI-15-mer, the final concentration of each reagent in the solution for the CL reaction was selected as follows: DFPO, 1.5 mmol/L; hydrogen peroxide, 5 mmol/L; TEA,  $2.5 \times 10^{-6}$  mol/L; *t*-BuOH, 25%.

Before performing a photographic detection, several kinds of the membranes were evaluated, using CBI-CHA as a standard sample. None of the nitrocellulose or nitrocellulose-based membranes could not be used, since they were soluble in DMP solution. Nylon membranes were found to exhibit a resistance to DMP solution and thus were employed. Among the membranes tested (Tropilon-Plus, Hybond-N+ and Zeta-Probe), Hybond-N+ gave relatively large background signals under the conditions tested; Tropilon-Plus and Zeta-Probe were selected for further investigation (Fig. 5).

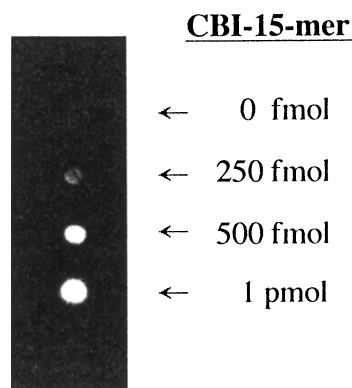
Fig. 6 demonstrates the results of photographic detections obtained using various concentrations of CBI-15-mer. Visual discrimination of the CL inten-



**Figure 4.** Effects of (a) hydrogen peroxide and (b) DFPO concentrations on CL intensity. The concentration of each compound in the CL reaction mixture was same as described in Fig. 1 except for various concentrations of (a) hydrogen peroxide and (b) DFPO



**Figure 5.** Photographic detection of CBI-CHA (100 fmol/spot) on various membranes



**Figure 6.** Photographic detection of CBI-15-mer on the membrane (Tropilon-Plus)

sities was feasible over the range examined, and as little as 250 fmol per spot of CBI-15-mer immobilized on Tropilon-Plus could be detected.

## CONCLUSIONS

In this paper, we have described fundamental studies on photographic detection of fluorescent-labelled oligonucleotide by PO-CL. The labelling reaction using NDA was performed within 10 min and the purification was easily performed by gel filtration followed by HPLC separation. Although the proposed photographic method is of less sensitivity than that with CL detection using an enzyme label (2), it includes simple procedures and thus might be applicable to the convenient detection of CBI-

oligonucleotide in dot-blot, Northern and Southern blot formats. In addition, the technique might be extended to include other fluorescent labels, e.g. fluorescein-labelled oligonucleotides and proteins, in membrane-based assays.

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