CHEMILUMINESCENCE, ANALYTICAL APPLICATIONS

1. Introduction

Chemiluminescence (CL) has many advantageous features as a tool of detection in instrumental analysis, including sensitivity, selectivity and simplicity. A CL reaction needs no excitation light source, thus, it is not accompanied with any scattering light. This results in a large signal-to-noise (S/N) ratio and consequently, a substantial increase in detector's sensitivity. To date, several kinds of CL reactions have been clarified on their reaction mechanisms. Generally, concentrations of the substrates or catalysts in each CL reaction can be determined by measuring the CL generated. Among the CL reactions, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) derivatives, acridine derivatives, aryloxalate derivatives, and ruthenium derivatives have frequently been utilized in analytical applications (1–4). In this article, representative analytical applications of CL in liquid phase for the determination of a variety of compounds are described.

2. Luminol Derivatives

2.1. Luminol as a CL Reagent. Luminol is generally used for the determination of compounds that catalyze the luminol CL reaction. Aliphatic alcohols, aldehydes, ethers, and sugars containing an oxygen atom in their molecules can be converted into H_2O_2 by oxygenation in photochemical reaction using anthraquinone disulfonate as a sensitizer, which can then be detected by a CL reaction with Co(II) and luminol (5). The lower limit of detection is in the picogram level. After separation on a cation-exchange column, Co(II) in rice powder was determined by high performence liquid chromatography (HPLC) in the same manner. The sensitivity is very high with the detection limit of 0.5 ng/L (6). Although Cu(II) can catalyze the luminol–hydrogen peroxide CL, the coexisting proteins can quench the yield of emission. Based on this phenomenon, as low as 50 ng of ovalbumin were determined by HPLC (7). Hydroperoxides, the primary

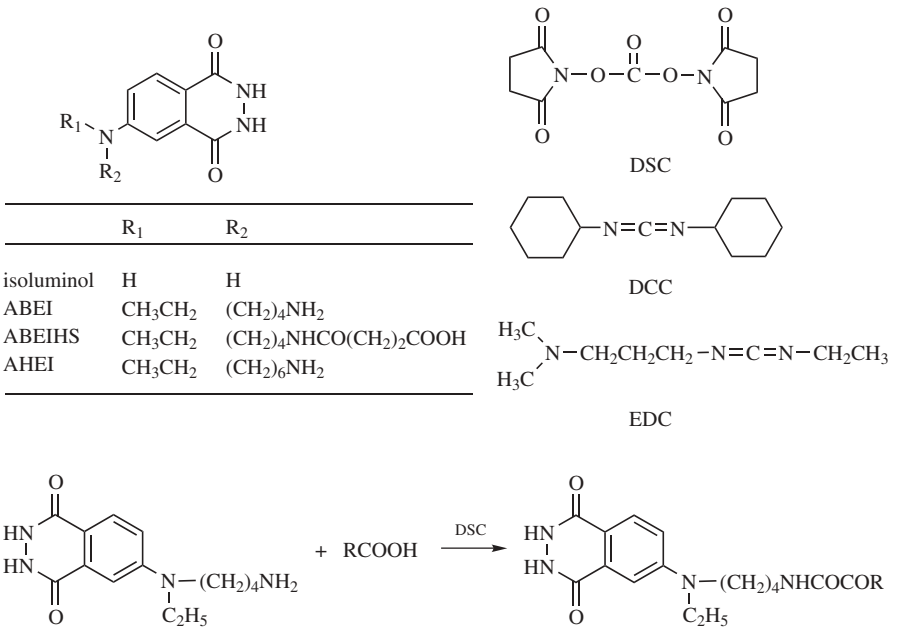
lipid peroxidation products, were separated on a normal-phase HPLC and selectively was determined by reaction with a mixture of luminol and cytochrome *c* as hydroperoxide-specific postcolumn CL reagents (8,9). Phosphatidylcholine derivatives are important biological phospholipids, which were separated on a normal-phase column and determined by the same CL reaction system; the amounts of peroxides were 28–431 pmol hydroperoxide–O_2/1 mL plasma (10). Phosphatidylcholine hydroperoxide in low density lipoprotein (LDL) in human plasma was determined by the same method, and revealed that the level is higher in patients with atherosclerosis and hyperlipemia compared to normal patients (11). Quantification of phospholipid hydroperoxides in biological tissues is important in order to know the degree of peroxidation damage of membrane lipids. Hydroperoxides of phosphatidiylcholine and phosphatidylethanolamine in rat liver and brain tissue were determined also (12). Determination of lipid peroxides in native LDL is clinically important because these peroxides are considered to cause the pathogenesis of atherosclerosis. A rapid flow injection method was developed by utilizing the luminol–microperoxidase CL reaction for the determination of peroxides in LDL (13). By using the same CL system, it was confirmed that the lipid peroxides in triacylglycerol contained in butter or spreads increased during the storage (14). The column switching HPLC method for simultaneous determination of hydroperoxides in phosphatidylcholine and cholesteryl ester has been developed; lipid hydroperoxide levels of phosphatidylcholine and cholesteryl ester in human plasma were 36.0 ± 4.0 and 12.3 ± 3.1 n*M*, respectively (15). Cholesterol hydroperoxide in human red cell membrane was also determined by a similar luminol–microperoxidase CL method (16). The unique CL method utilizing isoluminol and microperoxidase was developed for the measurement of lipid peroxidation in the cultivated medium, which can be used to directly measure *in vitro* lipid peroxidation in cells (17). The use of *m*-chloroperoxybenzoic acid instead of H_2O_2 causes an increase in luminol CL catalyzed by microperoxidase 8 by an order of magnitude. The enhancement of CL intensity is pH dependent (18). The manganese–tetrasulfonatophthalocyanine catalyzed by a luminol–H_2O_2 CL system, can be quenched in the presence of proteins. By using this quenching reaction, a highly sensitive method has been developed for the determination of proteins, such as human serum albumin, human γ-IgG, and bovine serum albumin (BSA) with the detection limits of 1.9, 2.7, and 3.4 ng/ml, respectively (19). Phenanthroline–H_2O_2 CL or luminol–H_2O_2 CL were used for the photographic determination of 1,10-phenanthroline and Fe(II) complex with the detection limit of 6×10^{-5} and 2×10^{-15} *M*, respectively (20).

2.2. Luminol Derivatives for the Measurement of Hydrogen Peroxide and Substrates that Produce Hydrogen Peroxide in Enzyme Reactions. Hydrogen peroxide is an excellent oxidizing agent for luminol; the CL increases with the increase of H_2O_2 concentration. Therefore H_2O_2 or substrates that produce it in an enzyme reaction can be determined by measuring the CL yielded; needless to say, enzyme activity can also be estimated. Glucose, cholesterol, and xanthines were determined by measuring H_2O_2 produced by enzyme reactions with glucose oxidase, cholesterol oxidase, and xanthine oxidase, respectively. Adenosine is converted to inosine by adenosine deaminase, which is further converted to hypoxanthine by nucleoside phosphorylase. The H_2O_2 formed by the

reaction of hypoxanthine and xanthine oxidase can be determined by the luminol–peroxidase CL reaction. This reaction system was applied to the automatic determination of purine derivatives; it is very rapid and 200 samples per day can be assayed (21). A flow injection (FIA)–CL method for the convenient determination of H_2O_2 was devised. In this system, an immobilized enzyme reactor (IMER) was placed in a flow line, and substrate was indirectly assayed by measuring the H_2O_2 yield. Moreover, FIA and HPLC methods for the determination of β, D-glucosides were developed; phenyl-β-D-glucoside, *p*-nitrophenyl-β-D-glucoside, and salicyl-β-D-glucoside are all converted to β, D-glucose with β, glucosidase, which was further converted into H_2O_2 with β-glucose oxidase. The H_2O_2 produced was determined by the luminol–CL reaction. Under the HPLC conditions employed, although, the mobile phase contained 30% acetonitrile, no remarkable influence on the activity of IMER was observed. The sensitivity of the method was 2 pmol on column as the detection limit (22).

The luminol CL reaction was also applied to immunoassays and DNA hybridization assays (23,24); by using microperoxidase and luminol as CL reagents, α-fetoprotein or ferritin as a tumor marker, thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were assayed. The HRP–luminol CL was utilized in hybridization assays of DNA on membrane, magnetic bead, polymer, etc. (25,26).

2.3. Isoluminol Derivatives as CL Reagents. Isoluminol has a higher luminescence efficiency than luminol. The 1-methoxy-5-methylphenadinium methylsulfate-isoluminol-microperoxidase CL reaction system has been utilized for the sensitive determination of 1-β- and 6-α-hydroxybile acids and bile acids in human urine samples. The detection limits of the method for bile acids were 8–250 pmol per assay. Among newborn babies, women, and women at a late pregnancy stage, the concentrations and conjugated forms of bile acids in their urine are markedly different, and the metabolic reaction of fetus and newborn babies are clearly different (27). A series of isoluminol derivatives has been synthesized for the chemiluminescent immunoassay (CLIA) by Schroeder and co-worker (28). These CL reagents have been applied to the assays of biotin and thyroxine and steroid hormones (estradiol, estriol, progesterone, etc). The CL reaction scheme is shown in Fig. 1. The labeling reaction of the analytes with these reagents needs condensation agents such as *N,N′*-disuccinimidyl carbonate (DSC), *N*-ethyl-*N′*-(3-dimethylaminopropyl)carbodiimide, and *N,N′*-dicyclohexylcarbodiimide. *N*-(4-Aminobutyl)-*N*-ethylisoluminol (ABEI) has been used as a precolumn labeling reagent in HPLC. For example, an unsaturated fatty acid, eicosapentaenoic acid, was labeled with ABEI using DSC as a condensing agent, and the derivative obtained was separated with a reversed-phase HPLC followed by CL detection with microperoxidase and H_2O_2 as postcolumn reagents. The determination range was 2 pmol–2 nmol on column with the detection limit being 200 fmol (29). Polyunsaturated fatty acids were determined by HPLC–CL using 6-[*N*-(4-aminobutyl)methylamino]-2,3-dihydro-1,4-phthaladinedione as a CL labeling reagent, EDC as a condensing agent, and a mixture of peroxidase–H_2O_2 as a postcolumn CL reagent. The DSC also can be used to condensate amino groups of reagent and analyte, which was used for the determination of amine compounds (30) and methamphetamine (31). *N*-(4-Aminohexyl)-*N*-ethylisoluminol (AHEI) was used for quantification of the *N*-terminal



	R_1	R_2
isoluminol	H	H
ABEI	CH_3CH_2	$(CH_2)_4NH_2$
ABEIHS	CH_3CH_2	$(CH_2)_4NHCO(CH_2)_2COOH$
AHEI	CH_3CH_2	$(CH_2)_6NH_2$

Fig. 1. Structures of isoluminol derivatives, condensation agents, and derivatization reactions of ABEI with carboxylic acid.

amino acid of peptides; 500 amol of the derivative can be detected (32). The distinct characteristics of ABEI analogues are that their reactions can be performed under the mild conditions, ie, at room temperature in polar solvents.

2.4. Other Luminol Derivatives. 4-Isothiocyanatophthalhydrazide and 6-isothiocyanobenzo[*g*]phthalazine-1,4-(2*H*,3*H*)-dione (IPO) were used as highly sensitive CL labels of amino compounds. Twelve amino acids were sensitively determined by HPLC with 4-isothiocyanatophthalhydrazide and a detection limit of 10 fmol on column was acheived (33). The antidepressant maprotiline in plasma could be detected at a very low level of 0.1 ng/ml by HPLC with IPO (34). Primary amines such as *n*-hexylamine, *n*-butylamine, and *n*-octylamine were sensitively detected using IPO as a label over the range from 30 to 120 fmol at a S/N of 3. Secondary amines such as *N*-methyloctylamine, di-*n*-amylamine, di-*n*-hexylamine of at levels of 0.8–3 fmol (S/N = 3) were determined using the same approach (35). A new luminol-type reagent, 6-aminomethylphthalhydrazide (6-AMP), was developed for the selective CL labeling of 5-hydroxyindole derivatives. As shown in Fig. 2, 5-hydroxyindoles were selectively reacted with 6-AMP in the presence of potassium hexacyanoferrate(III) to yield highly CL compounds. These compounds were detected after separation with a reversed-phase column by a postcolumn reaction with H_2O_2 and potassium hexacyanoferrate(III) in sodium hydroxide solution. The detection limits were as low as 0.7–4 fmol on column (S/N = 3) (36).

The principle that analytes of interest can be determined after being converted to luminol derivatives, was successfully introduced to the measurement of some biological components. For instance, 4,5-diaminophthalhydrazide

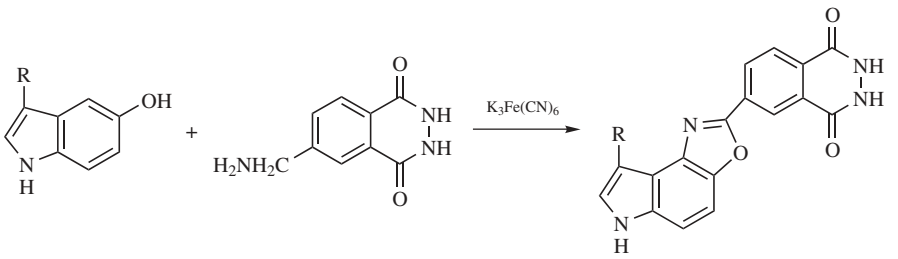


Fig. 2. Derivatization reaction of 5-hydroxyindole with 6-AMP.

(DPH) gives CL compounds by reaction with α-keto acids, which are important intermediates in the biosynthesis of amino acids, carboxylic acids, and sugars. Nine DPH derivatives of α-keto acids were separated by a reversed-phase column within 50 min followed by a postcolumn CL reaction with a mixture of H_2O_2 and potassium hexacyanoferrate(III) in sodium hydroxide solution; detection limits were in the range of 4–50 fmol on column. This method was applied to the assay of α-keto acids including phenylpyruvic acid; the detection limits were 9–92 pmol/mL plasma (S/N = 3) (37). The DPH was also applied to the quantification of *N*-acetylneuraminic acid derived from hydrolysis of sugar protein or sugar lipids in serum or urine; the detection limit was 9 fmol (S/N = 3) using as little as 10 μL of serum or 50 μL of urine (38). By modifying the reaction conditions, DPH can react with α-dicarboxylic acids to give CL quinoxaline derivatives. Phenylglyoxal, diacetyl, 2,3-pentanedione, 2,3-hexanedione and 3,4-hexanedione were examined and determined sensitively; the detection limits were 1.1–8.7 fmol except for 3,4-hexanedione whose limits were 300 fmol (39). 3α,5β-Tetrahydroaldosterone and dexamethasone have an α-keto moiety in their structure, which can be converted into a α-dicarbonyl moiety with cupper(II) acetate and determined with HPLC–CL using DPH as a derivatizing reagent (40). 5-Amino-4-sulfanilphthalhydrazide was synthesized as a CL labeling reagent for aromatic aldehydes in HPLC. Benzaldehyde, 4-tolualdehyde, 4-chlorobenzaldehyde, 4-formylbenzoic acid, 4-hydroxybenzaldehyde, and vanillin were selected as model aromatic aldehydes. The detection limits of these compounds were in the range of 0.2–4.0 fmol on column (41). 4-(6,7-Dihydro-5,8-dioxothiazolo[4,5-*g*]phtalazin-2-yl)benzoic acid *N*-hydroxysuccinimide ester as a sensitive CL reagent for amines was developed. Model amines such as methyl-*n*-octylamine, *n*-nonylamine, and *n*-decylamine were derivatized with this reagent followed by separation with a reversed-phase column and CL detection. The detection limits at S/N = 3 were subfmol levels on column (42).

2.5. Utilization of Enhancers for Luminol CL Reaction. In spite of the continuous efforts by analytical chemists to develop useful CL reagents, only a few new ones have been successfully developed. Thus, trials to enhance the efficiency of known CL reagents, were introduced in CL analyses. In the luminol–peroxidase–H_2O_2 CL system, phenol compounds such as 4-iodophenol (43), aromatic compounds such as hydroxybenzothiazoles and dehydroluciferins (44,45), and phenylboronic acids (46) have been found to serve as strong enhancers. Competitive CL immunoassay of *s*-Triazine pesticides with horseradish

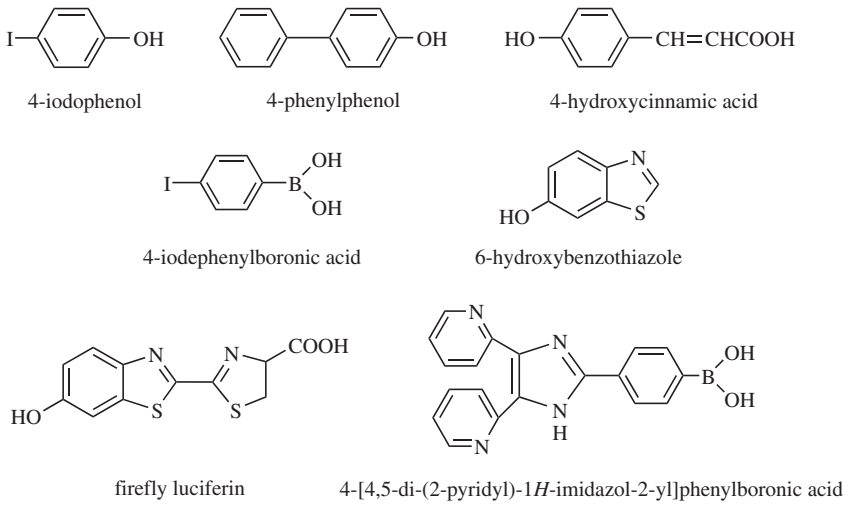


Fig. 3. Representative enhancers for luminol CL.

peroxidase as a label was developed by using 4-iodophenol as an enhancer. *s*-Triazine classification of environmental samples containing various analyte mixtures was correct in 79–100% of all cases depending on the type of analyte (47). Representative enhancers are summarized in Fig. 3. In the CL system using glucose oxidase as a labeled enzyme to produce H_2O_2, the CL efficiency can be increased by this enhancing system. Although the mechanism has not been clarified in details yet, it is proposed as follows where HRP is used as the enzyme. First, HRP accepts oxygen from hydrogen peroxide to form HRP-I and HRP-II, which withdraw hydrogen from luminol to give luminol radical; in this system, the enhancer accelerates the radical formation. The resultant radical may convert into the diazaquinone intermediate that produces an aminophthalic acid dianion and an emission of light by the reaction with H_2O_2. Substrates having 5-bromo-4-chloro-3-indolyl group (BCI) were synthesized for alkaline phosphatase (ALP), which were combined with this enhanced luminol CL. For instance, hybridization assays using ALP as a marker enzyme have been developed (48).

3. Acridine Derivatives

Lucigenin (*N*,*N*′-dimethyl-9,9′-biacridinium dinitrate), a representative chemiluminogenic acridine derivative, is one of the oldest CL reagents and has been used for analytical purposes. Lucigenin reacts with H_2O_2 to yield an emission of light in a similar manner to that of luminol, and it also can produce emission with organic reductants such as reducing sugars. This finding is based on the reaction of lucigenin with the α-hydroxycarbonyl group of the organic reductants. Therefore, compounds bearing an α-hydroxycarbonyl group such as glyceraldehydes, cortisols, phenacylalcohols, or pehancylesters can be determined by lucigenin

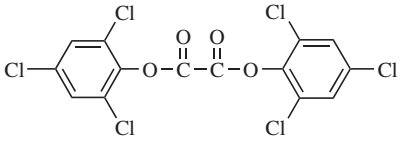
CL. A FIA method was developed for the determination of glucuronides; herein, IMER of β-glucuronidase hydrolyzed glucuronides to glucuronic acid, which was detected by the lucigenin CL reaction. Phenyl, nitrophenyl, methylumbelliferil, bromonaphthyl, estradiolglucuronide, and androsterone glucuronides were determined with the lower detection limits of 5–10 μM. The lucigenin CL detection system can be applied to HPLC with an ion-exchange column, where the disturbing effects of biological substances on quantification could be omitted (49). Corticosteroids and *p*-nitrophenacyl esters of 0.5-pmol levels on column were quantified by HPLC with lucigenin CL (50). Furthermore, lucigenin was found to yield an emission of light by reaction with catecholamines. By utilizing this phenomenon, catecholamines were quantified over the range of 1×10^{-7}–1×10^{-4} M (51). The lucigenin CL was applied for the investigation of human seminal plasma, which has powerful antioxidant and immunosuppressive activities (52). A sensitive and simple CL assay for alkaline phosphatase (ALP) was developed by using dihydroxyacetone phosphate or its ketal as a substrate. Both substrates were transformed to dihydroxyacetone by hydrolysis with ALP, followed by a CL reaction with lucigenin with the detection limits of 3.8×10^{-19} and 1.5×10^{-18} mol of ALP, respectively (53). Quantification of epinephrine with lucigenin CL was achieved by an FIA system fabricated on a microchip. Two types of flow cells were used; (*1*) two streams entered through separate inlet ports and merged to flow adjacently, and (*2*) a lucigenin solution-containing epinephrine was split up to 36 partial flows by passage through the nozzles, and was injected into the alkaline solution. The CL intensity in cell 2 was six-times larger than that in cell 1, with the detection limit of 8.0×10^{-7} M (54).

Acridinium esters or acid chlorides were developed as CL labeling reagents to be applied to immunoassays and HPLC of trace amounts of biological components. Acridinium labeled compounds have 100 times stronger CL intensity compared to luminol labeled ones, and acridinium esters have the dominant feature that they do not loss the CL efficiency even after binding to antigen or antibody. Acridinium esters, for instance, were applied to compensative DNA probe (cDNA) in hybridization assay (55), human gonadotropin and TSH (56), virus, and immunoassay of a cancer marker. By using a commercially available kit (TSA), as low as 0.01 mU/mL of TSH can be determined. This sensitivity makes it possible to clearly distinguish the TSH levels in normal human blood (0.53–3.05 μU) and Basedow's disease patients' blood (<0.10 μU) (57). 10-Methyl-9-acridinium carboxylate was used for HPLC–CL determination of the important environmental pollutants chlorophenols; the detection limits were very low of 300 amol–1.25 fmol (S/N = 3) (58). Several activated derivatives of 9-acridinecarboxylic acids were synthesized and their utility for H_2O_2 detection was investigated. Among these, 9-acridinecarbonylimidazole was found as a useful reagent for measuring the activity of a number of enzymes that directly produce peroxide, including glucose oxidase, with the detection limits in the 1–10-amol range (59). Brain natriuretic peptide (BNP) is a potent vasodilator and natriuretic factor regulating salt and water homeostasis. A nonradioactive sensitive and specific assay for N-terminus of the precursor of BNP was developed using 4-(2-succinimidyl-oxycarbonylethyl)phenyl-10-methylacridium 9-carboxylate fluorosulphonate as a CL label (60).

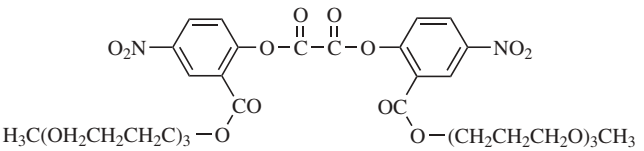
4. Aryloxalate Derivatives

4.1. Aryloxalates and Fluorescent Compounds as Chemiluminescent Reagents. Chemiluminescent produced by the reaction of aryloxalate (or oxamides), H_2O_2, and fluorescent compounds is known as peroxyoxalate chemiluminescence (PO–CL). Many kinds of aryloxalates have been synthesized and evaluated as PO–CL reagents. Among these, bis(2,4,6-trichlorophenyl)oxalate (TCPO) is the most popular. The TCPO can be easily synthesized and is inexpensive. The solubility of this compound is low in organic solvents such as acetonitrile and methanol commonly used in HPLC, which may limit its use in some cases. Meanwhile, bis[2-(3,6,9-trioxadecyloxycarbonyl)-4-phenyl]oxalate (TDPO) was found to be useful because of its higher CL efficiency and greater solubility in organic solvents compared to TCPO. In PO–CL, fluorescent compounds having large fluorescent quantum yields and lower oxidative electric potentials to yield efficient CL intensities are preferable. Representative aryloxalates are presented in Fig. 4.

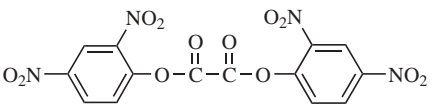
4.2. Determination of Fluorescent Compounds. Generally, highly fluorescent compounds, which should be suitable to PO–CL detection, are rare among biologically active components, environmental pollutants, etc. Therefore, derivatization of non-fluorescent or weakly fluorescent compounds to yield highly fluorescent ones is commonly adopted practice in PO-CL. Many known fluorescence derivatization methods can be used to this aim. Dipyridamole, a blood platelet improving agent, and benzydamine, an antiinflammatory agent, in rat plasma samples were sensitively determined by PO–CL without derivatization, because they have strong native fluorescence in themselves (61). Phenothiazine antipsychotic agents are also determined without derivatization (62). Many aromatic compounds such as air pollutants have strong native fluorescence, and



TCPO



TDPO



DNPO

Fig. 4. Representative aryloxalates are TCPO, TDPO, and bis(2,4-dinitrophenyl)oxalate (DNPO).

thus, can also be determined with PO–CL without a derivatization step. A FIA method for determining perylene, anthracene, and pyrene was developed using TCPO and H_2O_2 in acetonitrile as CL reagents with the detection limits of 0.05, 65, and 75 pg (S/N = 5), respectively (63). Nitroarenes derived from diesel engine exhaust are known as carcinogens. In spite of being non-fluorescence, nitroarenes can be determined after reduction to highly fluorescent aminoarens. Air pollutants such as polycyclic aromatic hydrocarbons (PAHs) and their nitrated derivatives were determined over a period of 12 months by HPLC–PO–CL. The toxic equivalency factors adjusted concentration of total PAHs determined was 2.33 ng/m^3 in Nagasaki city area (64). Fluorescent compounds having their absorption wavelengths in the infrared (ir) region are preferable as fluorophores, because they can be excited with low excitation energy that generally the PO–CL reaction can produce. Methylene blue, pyridine 1, oxazine 1, and 3,3′-diethylthiadicarbocyanine iodide (DTDCI) were evaluated as fluorophores in HPLC–PO–CL, and were sensitively detected with the detection limits of 120, 27, 31, and 0.19 fmol on column, respectively (65). Among these, DTDCI is a potential candidate as a lead compound for preparing fluorophores in PO–CL.

A photographic method was devised for the detection of FL compounds and H_2O_2. Pyrimido[5,4-*d*]pyrimidine derivatives and rhodamine B can be detected as colored spots on an instant photographic film. Glucose in serum was semiquantified by the method (66). The CL immunoassay of antihuman T-cell virus antibody was developed by a photographic method (67).

4.3. Determination of Amino Compounds. Dansyl chloride (Dns-Cl) was first used for FL labeling of amino acids in thin-layer chromatography (TLC) (68). Excitation energy for Dns-derivatives is usually covered with that of PO–CL reaction. Sensitivities for determining several kinds of primary alkyl amines in PO–CL were compared using some labeling reagents, ie, Dns-Cl, 4-chloro-7-nitrobenzo-1,2,5-oxazole (NBD-Cl) and *o*-phtalaldehyde (OPA). The detection limits of DNS-, NBD- and OPA-amines were 0.8–14, 19–270, and 94–580 fmol, respectively (69). Dns-amino acids were also quantified by HPCL–PO–CL using TCPO as an oxalate reagent with the detection limit of 10 fmol (70). Sixteen kinds of Dns-amino acids were separated within 30 min by a gradient elution, and detected over the range from 2 to 5 fmol (S/N = 2). By applying a microbore column in this method, as low as 0.2 fmol of dansylated Ala, Val, Ileu, and Phe could be detected (71).

Furthermore, Dns derivatization was applied to quantifiy abuse drugs (amphetamines) in urine samples with the detection limit of 2×10^{-10} *M* (72). Methamphetamines in a single hair sample were detected at very low level of 20 pg (73).

Mexiletine, an antiarrhythmic drug, was assayed in rat plasma using TDPO as an oxalate over the calibration range of 20–100 ng/mL with the detection limit of 1.0 fmol on column (74). A highly sensitive HPLC–PO–CL method for catecholamines, which act as neurotransmitters, was developed using fluorescemine as a label with the detection limit of 25 fmol (75). The sensitivity is 20-times higher than that of FL detection and only 10 μL of urine sample were needed for quantification. Meanwhile, it was reported that FL detection was superior than CL detection in HPLC for histamine. Fluorescamine derivatives of histamine can be detected with FL 100 times more sensitively than

CL (76). This large difference may be due to the limitation of solvents to separate fluorescamine derivatives in HPLC–PO–CL, where solvents seriously affect the CL efficiency. Fluorescamine has also been utilized to quantify sulfamethazine (an antibacterial drug) in chicken eggs and serum; 1 ng/mL of standard sulfamethazine can be detected (77). 4-(*N,N*-Dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) is non-fluorescent, but gives strong fluorescent derivatives by the reaction with primary and secondary amines. Methamphetamine and its related compounds were simultaneously assayed by HPLC with TDPO and H_2O_2 as the postcolumn CL reagents. Six DBD-derivatives were separated by a reversed-phase column with a gradient elution, and their CL intensities were monitored over the range from 25 to 133 fmol (S/N = 3) by using DBD-*N*-ethylbenzylamine as an internal standard (78). The DBD-F was also utilized for determining metoprolol in serum (79). Naphthalene-2,3-dicarboxaldehyde (NDA) reacts with primary amines to yield fluorescent cyanobenz[*f*]isoindole (CBI) derivatives (80). By utilizing this labeling reaction, dopamine and norepinephrine in urine were determined at sub–low femtomole levels with a sample size of only 20 μL (81). Fluvoxamine, an antidepressant, was also determined with the same manner (82). Ethylenediamine was applied for the on-line postcolumn derivatization approach of catecholamines following PO–CL detection with TDPO and H_2O_2 as the postcolumn reagents. The method is very sensitive and allows the detection of catecholamines as low as 1 fmol on column (S/N = 2). Furthermore, the method was applied to develop an automatic analysis of catecholamines in rat plasma (83). The automatic analytical method was applied to determining (−)-isoproterenol and (−)-(*R*)-1-(3,4-dihydroxyphenyl)-2-[(3,4-dimethoxyphenethyl)amino]ethanol in rat plasma with the detection limits of 1.3 and 0.9 fmol on column, respectively (84). 1,2-Diarylethylenediamine derivatives can also be used for precolumn labeling of catecholamines. The labeled compounds were separated on a reversed-phase column and detected by PO–CL with TDPO and H_2O_2 as CL reagents; amol levels of catecholamines could be detected at S/N = 3 (85). Luminarin 1 bearing quinolizino coumarin skeleton as a fluorophore and *N*-hydroxysuccinimide ester as a reactive group, reacts with amines to give highly fluorescent compounds. Luminarin derivatives of pentylamine, pyrrolidine, thiamine, and proline were separated on a reversed-phase column and detected by PO–CL with the detection limits of 15–100 fmol (S/N = 3), which were 3–10 times more sensitive than those obtained by fluorescence detection (86). Representative derivatization reagents for amines are summarized in Fig. 5.

Sensitizing effects of polyamines on CL reaction was utilized to their sensitive determination in tomatoes. A FIA method was developed for determining 55 kinds of amines by using TDPO and sulforhodamine as CL reagents; aliphatic amines of $\sim 1.2 \times 10^{-8}$ *M* and polyamines of $\sim 7 \times 10^{-10}$ *M* were detected. Histamine in fish meat samples was determined by the same method (87).

4.4. Carbonyl Compounds. 5-*N,N′*-Dimethylaminonaphthalene-1-sulfonohydrazide (Dns-H) was used as a labeling reagent for carbonyl compounds. Oxo-steroids and oxo-bile acid ethyl esters in serum samples were derivatized to Dns-hydrazones, which were purified by gel-permeation chromatography, separated on a reversed-phase columns, and detected with TDPO–PO–CL. Corticosterone, testosterone, and progesterone were quantified with the detection limits of 3, 2, and 4 fmol at S/N = 2, respectively (88). 7α-Hydroxy-3-oxo-5β-cholanic

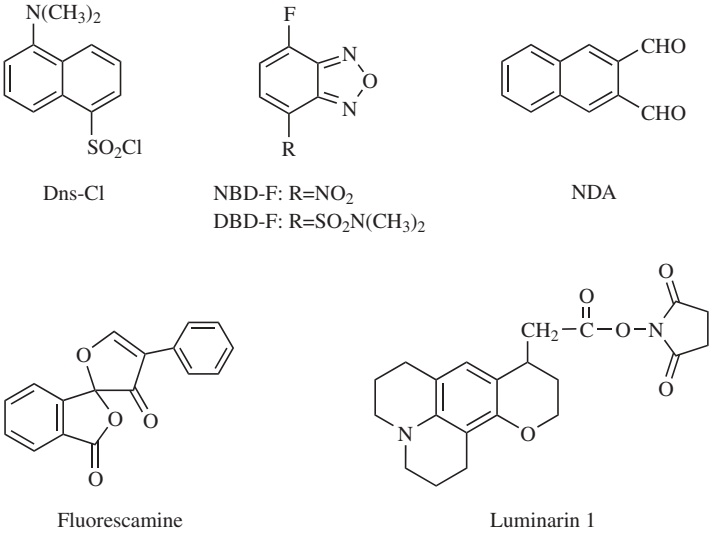
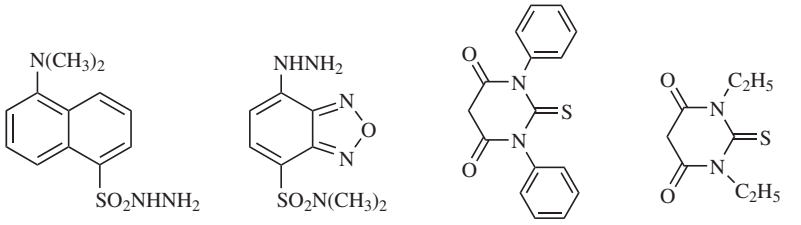


Fig. 5. Labeling reagents for amines in CL detection.

acid, an unusual oxo-bile acid, in cholestatic hepatic disease patient's urine sample was determined at nmol/L level. 3α- or 3β-Hydroxysteroide, 3β-hydroxy-5-cholenic acid, pregnanediol, 5-pregnene-3β,20β-diol, 5-pregnene-3β,20α-diol were converted into 3-oxo-steroide with the IMER of hydroxysteroidedehydrogenase, which was derivatized with Dns-H followed by reversed-phase separation and CL detection at a few femtomole level (89). Dns-H was also applied to the labeling of sugars; hyaluronic acid, chondroitin sulfate, dermatan sulfate were determined with the detection limit of 100 fmol (S/N = 3). Hyaluronic acid was converted with hyaluronidase SD to an unsaturated disaccharide, ΔdiHA, and labeled with Dns-H to give a FL compound. Disaccharides in rat's mast cells can be determined (90). Carbonyl compounds in air such as formaldehyde, acetaldehyde, and acetone were determined with HPLC–PO–CL after labeling with Dns-H (91). These compounds were derivatized by drawing an air sample through a small glass cartridge packed with porous glass particles impregnated with Dns-H; this method is very simple and can detect amounts at sub-ppb concentrations of carbonyl compounds. 4-(*N,N*-Dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H) showing very weak FL, reacts with carbonyl compounds to yield strongly FL derivatives. Medroxyprogesterone acetate, a synthetic progesterone, reacts with DBD-H to form a fluorescence compound, whose determination in serum was performed in the calibration range of 15.6–96.6 ng/mL using 100 μL of sample (92). Propentofylline, which has a nervous cell protecting effect, was quantified by TDPO–PO–CL with 0.031 ng on column (93). Microdialysate obtained from rat hippocampus was successfully applied to this study. 3-Aminofluoranthene was applicable to labeling of aldehydes and ketones (94). Malondialdehyde is well known as a degradation product of polyunsaturated lipids, and has been used as a marker of biological lipid-peroxidation. 1,3-Diphenyl-2-thiobarbituric acid (DPTBA) and 1,3-diethyl-2-thiobarbituric acid (DETBA) were proposed as useful labeling reagent candidates



Dns-H DBD-H DPTBA DETBA

Fig. 6. Labeling reagents for carbonyl compounds in CL detection.

for malondialdehyde (MDA). By using DETBA, MDA in fractions of rat brain tissues was first determined with TCPO–H_2O_2 CL (95). Labeling reagents for carbonyl compounds used in PO–CL are summarized in Fig. 6.

4.5. Determination of Carboxylic Acids. Coumarin derivatives, ie, 4-(bromomethyl)-7-methoxycoumarin (Br-Mmc), 7-(diethylamino)cumarin-3-carbohydrazide (DCCH), 7-(diethylamino)-3-[(4-iodoacetylamino)phenyl]-4-methylcumarine (DCIA), have been used for labeling of carboxylic acids. These reagents were evaluated as PO–CL using a TCPO–H_2O_2 CL reagent. However, Br–Mmc was found to give non-CL derivatives, because the energy of the PO–CL system used was not enough to excite them. Meanwhile, DCIA derivatives of linear-chain fatty acids showed good CL with low femtomole levels of detection limits (96). *N*-(Bromoacetyl)-*N*′-[5-(dimethylamino)naphthalene-1-sulfonyl]piperadine (Dns-BAP) was developed as a labeling reagent for carboxylic acids, and applied to their HPLC–PO–CL assays. For instance, carboxylic acids were derivatized in aprotic solvents at 55°C for 30 min under coexistence of 18-crown-6 and potassium bicarbonate, followed by separation on a reversed-phase column and detection with bis(2-nitrophenyl)oxalate and H_2O_2 as CL reagents. Retinein acid, a precursor of vitamin A, was also detected at 25 fmol (97). 6,7-Dimethoxy-1-methyl-2(1*H*)-qinoxalinone-3-propionyl carboxylic acid hydrazide was applied to determine metabolites of arachidic acids with EDC and DCC as condensing reagents. The derivatives were determined by HPLC with TCPO–H_2O_2 as CL reagents at 500 amol (S/N = 3) (98). 2-(4-Hydrazinocarbonylphenyl)-4,5-diphenylimidazole (HCPI) can be applied to fluorescent labeling of fatty acids. The HCPI derivatives of fatty acids in human serum were detected by HPLC–PO–CL over the range of 12–18 fmol (S/N = 2) (99). 7-(Diethylamino)coumarin-3-carbohydrazide and luminarin 4 can also be applied to determine sensitively fatty acids at femtomol levels (100).

Derivatizing reagents for carboxylic acids used in PO–CL are shown in Fig. 7.

4.6. Determination of Thiols, Alcohols, Phenols and Others. Usable fluorescent derivatizing reagents for thiols and phenols are limited. *N*-[4-(6-Dimethylamino-2-benzofuranyl)phenyl]maleimide (DBPM), a maleimide-type fluorescent reagent, was used for thiol compounds. The DBPM as well as other maleimide-type fluorescent labeling reagents is natively non-fluorescent, but gives strong fluorescent products upon reaction with thiol compounds. The DBPM derivatives of biologically important thiols such as glutathione, cysteine, *N*-acetylcysteine, cysteamine, and antirhumatide D-penicillamine were separated

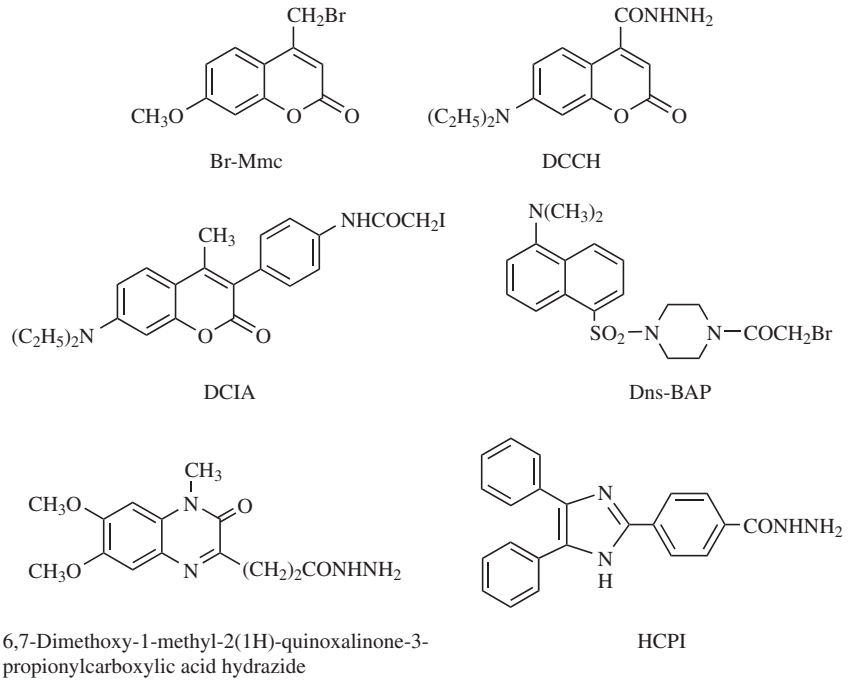
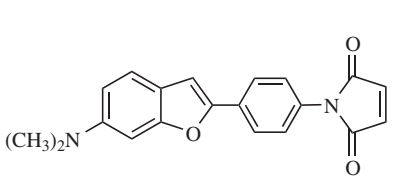


Fig. 7. Labeling reagents for carboxylic acids in CL.

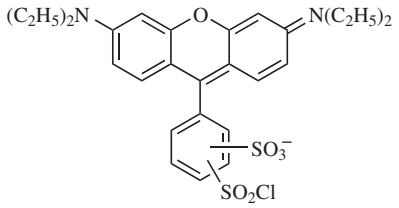
on a reversed-phase column and detected by PO–CL using TCPO and H_2O_2 as the postcolumn reagents. The derivatives were separated within 12 min, and detected in the range from 7 to 113 fmol (S/N = 2) on column (101). The method was applied to the analysis of glutathione and cysteine in rat liver tissue.

Normal phase separation is preferable to a PO–CL detection system, because PO–CL can yield a stronger emission of light in nonaqueous solvents than in aqueous solutions. However, due to the limited usability of normal-phase separation for biological samples, only a few reports have appeared. Estradiol (one of the major estrogens secreted by the human ovary) in serum was determined after extraction by a solid-phase column followed by Dns-derivatization and separation on a normal-phase column. The method can detect as low as 50 pg of estradiol (102). Estradiol in plasma sample was also determined by HPLC–PO–CL combined with liquid–liquid extraction using ethyl acetate. The detection limit of Dns–estradiol was 15 fmol (4 pg) in the standard solution and 44 fmol (12 pg) in the rat plasma (103).

Phenols labeled with lissamine rhodamine B sulfonyl chloride show high quantum yield and emission at long wavelength (>550 nm), which permits a considerable reduction of the background emission. The derivatization can be performed under mild conditions, ie, at room temperature for 1 min. Lissamine Rhodamine B derivatives of chlorophenol was detected in the low picogram range after normal or reversed-phase column separation following PO–CL detection. Pentachlorophenol in river water could be detected as low as 0.8 ppb without pretreatment (104). DBPM and lissamin rhodamine B sulfonyl chloride are shown in Fig. 8.



DBPM



Lissamin rhodamine B sulfonyl chloride

Fig. 8. *N*-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide (DBMP) and lissamin rhodamine B sulfonyl chloride.

The DCIA, a labeling reagent for carboxylic acid, can be also applied to fluorescent labeling of fluoropyrimidines anticancer drugs using a crown ether–potassium complex as a catalyst. The DCIA derivatives of 5-fluorouracil, 5-fluorouridine, 5-fluoro-5′-deoxyuridine, and 1-(tetrahydro-2-furanyl)-5-fluorouracil were detected at several tens of a femtomole level (105,106). Metal ions can be determined after formation of chelates with 8-hydroxyquinoline followed by FIA or HPLC with PO–CL detection. The ions Al(III), Zn(II), Cd(II) and In(III) were determined with the detection limit from 20 to 70 ppb (107).

PO–CL can also be applied to the detection of oxalic acid and porphyrins (108,109). Oxalic acid emits light in ethanol solution by the reaction with H_2O_2, *N*,*N*′-dicyclohexylcarbodiimide, and diphenylanthracene at pH 1. Oxalic acid in serum was also quantified. Enhancement of this PO–CL by porphyrins was applied to their determination in urine.

A capillary electrophoresis (CE) with PO–CL detection was developed for Dns–amino acids, which is 35 times more sensitive than ultraviolet (uv) detection (110). Bovine serum albumin forms complexes with eosin and rose bengal, which can be separated by CE. These complexes were analyzed after separation by CE followed by PO–CL detection; albumin, from 5×10^{-7} to 1×10^{-4} *M*, can be determined with the detection limit of 2×10^{-7} *M* (4 fmol) (111). The sensitivity of the method was further improved to be 1.7 fmol (112). The CE, thus, will become a useful tool for analysis of macromolecular biological compounds (113–115).

4.7. Determination of Hydrogen Peroxide and Substrates that Produce Hydrogen Peroxide in Enzyme Reactions. Hydrogen peroxide and substrates from which H_2O_2 is produced in enzymatic oxidation can be sensitively determined by using a PO–CL system. Several enzymatic oxidation reactions are summarized in Fig. 9. For these aims, batch, FIA, HPLC, photographic methods, etc, have been developed. For the determination of H_2O_2 by PO–CL, pyrimido[5,4-*d*]pyrimidines were synthesized and evaluated. Among them, 2,4,6,8-tetrathiomorphorinopyrimido[5,4-*d*]pyrimidine (TMP) was found to give the largest CL (116). Structure of TMP is given in Fig. 10. As low as 1×10^{-8} *M* of H_2O_2 was quantified by a batch method with TCPO and TMP as reagents (117). The H_2O_2 was first determined by HPLC after separation on reversed-phase column followed by CL detection with TCPO and TMP as reagents with the detection limit of 188 fmol (9.9×10^{-9} *M*) at S/N = 3. Hydrogen peroxide in

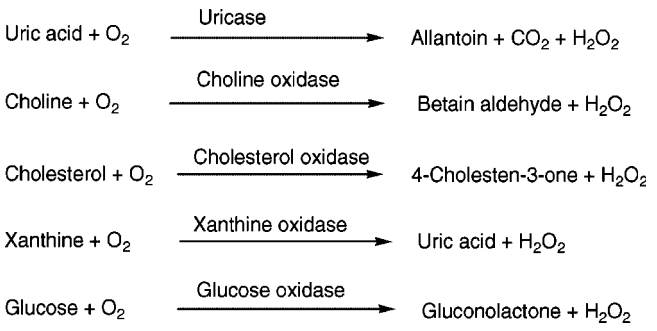
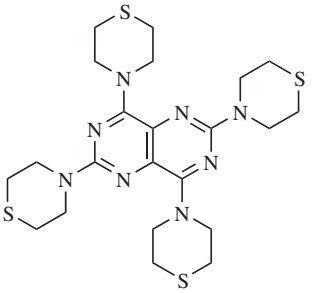


Fig. 9. Representative enzyme reactions that produce hydrogen peroxide.

coke drinks and commercially available hydroperoxide reagents were successfully determined by this method (118). Rhodamine derivatives can increase the PO–CL efficiency. A FIA method using sulforhodamine 101 as a sensitizer can detect as low as 3×10^{-9} *M* H_2O_2 with TDPO as an oxalate (119). A fluorescent reactor of 3-aminofluoranthene immobilized on pore glass beads was used in FIA, and H_2O_2 could be detected at 1×10^{-8} *M* (120).

An IMER can be used as an online reagent for the determination of substrates in FIA or HPLC system. However, several conditions should be optimized to obtain a satisfactory CL yield, ie, nature, pH, and flow rate of carrier, reaction temperature, catalysts, CL reagents, organic solvents, etc. Eight kinds of L-amino acids were enantio-selectively determined by using an IMER of L-amino acid oxidase in urine and beer (121). The IMERs of glucose oxidase and uricase were applied for quantification of glucose and uric acid, respectively. These methods are simple and can be applied to human serum without pretreatment except a simple dilution. An IMER immobilized with acetylcholine esterase and choline oxidase was applied to determine simultaneously acetylcholine, an important neurotransmitter, and choline. The determination range is from



TMP

Fig. 10. Structure of TMP.

10 pmol to 10 nmol on column (122). Phospholipids containing choline were determined by a hyphenated HPLC–FIA method. Phospholipids are highly lipophilic compounds, and thus, a special column such as an aminopropyl-modified silica gel column is required for their separation. Several kinds of choline containing phospholipids were separated and fractionated. Each fraction was dried *in vacuo* and redissolved in Triton-X aqueous solution, and the resulted solution was injected into FIA–PO–CL system equipped with the IMER of phospholipase D and choline oxidase. Choline-containing phospholipids in human serum can be determined by this method (123). Polyamines are regarded as cancer markers, which can be determined by HPLC–PO–CL. After separation on a reversed-phase column, polyamines were converted into H_2O_2 by the IMER of polyamine oxidase and putrescine oxidase. Putrescine was detected with DNPO and 8-anilinonaphthalene sulfonic acid as CL reagents with the detection limit of 5 pmol (124). In the same manner, polyamines and their monoacetylated derivatives can be determined by using the IMER of acetylpolyamine amidohydrolase and putrescine oxidase with TCPO and TMP as CL reagents. The method was applied to determine polyamines in potatoes (125). Pyruvic acid can be converted to H_2O_2 by the IMER of pyruvic acid oxidase, which was determined by PO–CL with TCPO and perylene as the postcolumn reagents. Pyruvic acid in serum can be quantified over the range of 1×10^{-6}–1×10^{-2} *M* by this method.

5. Dioxetane Derivatives

Adamantyl dioxetanes have been used as analytical reagents of CL. 3-(2′-Spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)-1,2-Dioxetane (AMPPD) emits of light by the decomposition with alkaline phosphatase (ALP). By utilizing this reaction, as low as sub-attomol levels of ALP were assayed (126). The AMPPD can be successfully applied for enzyme immunoassay with LAP as a label by an automatic analyzer. In the same manner, 3-(2-spiroadamantane)-4-methoxy-4-(3″-β-D-galactopyranosyl-oxyphenyl)-1,2-dioxetane (AMPGD) is decomposed with β-D-galactosidase (β-Gal) to emit light, by which low levels of β-Gal can be determined. Several kinds of enzymatic substrates having adamantly dioxetane as an emitter have been developed for β-glucuronidase, β-glucosidase, and phospholipase. Owing to the very long lifetime of CL produced by adamantly dioxetanes, they are recommended to photographic detection. Reverse transcriptase (RT) assays are very important for the detection of retroviruses including human immunodeficiency virus (HIV) and for the development of new antiretroviral substances. A highly sensitive RT assay was developed by using 0.25 mM disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2′-(5′-chloro)-tricyclo[3.3.1.1.3,7]decan]-4-yl)phenyl phosphate (CSPD) and 0.1 mg/mL poly(benzyltributyl)ammonium chloride in 1 *M* diethanolamine buffer (pH 9.5) containing 1 m*M* $MgCl_2$ as a CL reagent and enhancer solution. The assay can detect the RT activity in serum, plasma, and cell culture medium directly without preconcentration or extraction of enzyme (127). A sensitive CL enzyme-linked immunosorbent assay (ELISA) for the bioanalysis of carboxy-terminal B-chain analogues of human insulin was developed using only 10 μL of serum sample (128).

6. Lophine and Indole Derivatives

Lophine (2,4,5-triphenylimidazole) is the first synthetic CL compound. However, its analytical applications are relatively rare. Lophine produces an emission of light by the reaction with H_2O_2, which is sensitized by some ions such as Co(II), ClO^-, Cr(III), and Cu(II). These ions, thus, can be determined by measuring the CL produced at the concentration of $\sim 10^{-6}$ *M*. Hydroxylamine was found to enhance the CL of lophines-H_2O_2-Co(II) reaction, and by a FIA method with this enhancing reaction, CL intensities of 18 kinds of lophine derivatives were evaluated (129). Furthermore, this enhancement was utilized for quantification of Co(II) with the determination limit of 4.5×10^{-8} *M* (130). Lophines show very strong FL, and thus they have been applied to FL detection rather than CL detection.

Indole derivatives have been much studied on their CL mechanisms because their structures are well correlated with luciferin of *Cypridina hilgendorfi*, but studies on their analytical characteristics are rather few. Indoxyl β-D-galactose was synthesized as a substrate of β-Gal, which allowed the assay of as low as 3 amol of β-Gal (131). Superoxide-triggered CL of *Cypridina* luciferin analogue, 2-methyl-6-phenyl-3,7-dehydroimidazo[1,2-α]pyrazin-3-one, is enhanced by nonionic detergents, such as Tween 20, Tween 80, and Triton X-100. By utilizing this detergent-amplified CL reaction, xanthine and xanthine oxidase were determined as low as 5 nmol/L and 3.85×10^{-7} U/mL, respectively (132).

7. Ruthenium(II) Complex

Tris(2,2′-bipyridyl)ruthenium(II) complex (bpy = 2,2′-bipyridyl) is a strongly fluorescent compound, which emits at 620 nm with excitation of 450 nm. As shown in Fig. 11, CL is produced by the oxidation of ruthenium(II) complex and reducing compound on the surface of the electrode followed by the excitation of oxidized ruthenium(III) complex with a radical derived from reducing compound. Therefore, reducing compounds can be determined after HPLC separation and following oxidation. Primary amines yield little CL on this system, but secondary and tertiary amines, especially cyclic aliphatic amines can

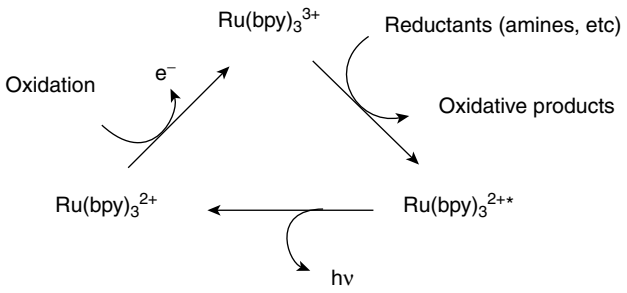


Fig. 11. Electrogenerated chemiluminescence (ECL) reaction of ruthenium(II) complex with reductants.

produce strong CL. The CL intensities of aliphatic amines are ordered as follows: tertiary > secondary > primary, and thus, many kinds of medicines containing a tertiary aliphatic amine moiety were determined by this system. Primary amines can be sensitively determined after conversion into tertiary amines; propylamine and 3-aminopentane were determined after conversion to the corresponding cyclic tertiary amines with divinyl sulfone followed by reversed-phase separation and ECL detection with the detection limits of 30 pmol and 1 pmol on column, respectively. In the same manner, a simultaneous determination of primary, secondary, and tertiary amines was performed; after an HPLC separation, primary and secondary amines were converted to tertiary amines with acrylonitrile and detected by ECL (133). A new electrogenerated chemiluminescent immunoassay (ECLIA) was developed for the determination of cytokeratin 19 (CYFRA 21-1), a marker of epithelial differentiation, in serum and urine samples. The clinical value of urinary CYFRA 21-1 for the detection of bladder cancer was evaluated (134). D- and L-Tryptophan were determined after a ligand-exchange HPLC followed by ECL detection with 0.2 pmol on column (S/N = 2) (135). Dns-amino acids were also determined by ruthenium(II) complex CL; Dns–Glu was quantified with the detection limit of 0.1 μM (2 pmol, S/N = 2), which is 1000 times more sensitive than that of nonlabeled Glu (136). Oxalic acid in urine and blood samples was assayed after a separation with a reversed-phase ion-paired column followed by ECL detection with the detection limit of 1 nmol/mL (25 pmol on column) (137). By directly adding ruthenium(II) complex to the mobile phase, the detection limit can be improved to 0.1 μM, which was applied to the assay of erythromycin in urine and plasma samples with the detection limit of 50 fmol on column (S/N = 3) (138). Similarly, a new type of erythromycins, de(*N*-methyl)-*N*-ethyl-8,9-anhydroerythromycin A 6,9-hemiacetal and its three metabolites in plasma and urine samples were quantified with the determination limits of 1 and 10 ng/mL, respectively (139). Glyphosate, a herbicide, and its structural analogues were determined after HPLC separation and ECL detection, and their CL intensities were ordered as follows: glycine < diethanol amine < hydroxyethyl glycine < iminodiacetic acid < glyphosate. The detection limit of glyphosate was 0.01 μM (140). Yohimbine (an indole alkaloid with α_2-adrenergic blocker) in human serum was separated with an ion-paired column and determined by ECL with the detection limit of 30 ng/mL (S/N = 3) (141). Tris(1,10-phenanthroline)ruthenium(II) complex (phen = 1,10-phenanthroline) was used as a CL reagent and applied to determine oxalic acid in tea (142).

Labeling reagent was prepared by introducing *N*-hydroxysuccinimide into ruthenium(II) complex, which can be used for ECL immunoassay of antigens, antibodies, and nucleic acids. An automatic analyzer using ECL immunoassay is commercially available, and utilized for clinical analysis of markers of hepatic cancer or infectious diseases.

8. Others

In spite of its long history, gallic acid has a few analytical applications. Recently, catechin and (−)-epigallocatechin 3-gallate, which are focused as a radical quencher, were found to yield CL by the reaction with acetaldehyde, horseradish

peroxidase and H_2O_2. Catechin in rat and human plasma samples was assayed by this method (143). Pyrogallol is also known as a potential CL compound. Carix[4]allenes having pyrogallol as a structural unit were synthesized, and their CL properties were examined (144). The analytical applications of these compounds are anticipated.

Adenine and guanidine compounds can produce an emission of light by reaction with 9,10-phenanthrenequinone, which could be utilized for HPLC–CL of guanidino compounds (145). As well as adenine and its nucleosides and nucleotides (146,147), guanine and its nucleosides and nucleotides (148) react with glyoxal derivatives to give CL compounds, which can intensely emit under alkaline conditions in aprotic solvents such as dimethylformamide (DMF). For instance, adenyl compounds react with dimethylglyoxal dimethylacetal to give strong CL in the coexisting of tungustic acid and propanol; as low as 3×10^{-8} *M* of adenine, which corresponds to a few nanogram of DNA can be detected (149). 3′,4′,5′-Trimethoxyphenylglyoxal can yield intense CL by the reaction with DNA or guanidine nucleotide. The DNA adsorbed on a nylon membrane was sensitively detected at zmol by a CCD camera at $-25^{\circ}C$ (150).

The CL of nitrogen monoxide (NO) occurs with the reaction of ozone in the range of 600–3200 nm. Nitrogen monoxide produced from NO_2^- by the reduction with potassium iodide was analyzed by FIA or HPLC with the detection limit of 0.1 ng (151). After nitrosation of glyphosate in grain, the resultant *N*-nitrosoglyphosate was separated by an ion-exchange column, and NO derived by a denitrosation reaction was detected. The method was applied to the determination of glyphosate in water, beer, ale, lentils and beans, and a few cereals (152). Purity of the pharmaceutical-grade synthetic peptides was evaluated by using a nitrogen–CL detector (153). *N*-Nitroso compounds (NOC) comprise nitrosoamines and nitrosamides, and were produced by the nitrosation of secondary amines and *N*-alkyl amides, respectively. Total NOC and their precursors were found in extracts of food and tobacco products. After these compounds were decomposed to NO with refluxing HBr/HCl/HOAc/EtOAc, NO was measured by CL (154).

Morphine, a narcotic analgesic, can yield CL by reaction with potassium permanganate in polyphosphoric acid solution (pH 1–2). After separation with a stylene–divinylbenzene column, CL produced from on-column reaction of morphine with potassium permanganate was assayed with the detection limit of 0.5 ng on column (155). Similarly, morphine and monoacetylmorphine were simultaneously determined with the detection limits of 20 and 300 pg on column, respectively (156). The 1,10-phenanthroline–H_2O_2–Cu(II) CL system was applied for assay of BSA. Gradient elution as a function of pH with a metal-chelate affinity column was examined for the separation of BSA, lysozyme, and bovine serum γ-globulin in the range from 1×10^{-4} to 1×10^{-1} g/L; the sensitivity is 200 times higher than that with uv detection (157).

In a similar manner, ovalbumin-trypsin inhibitor and BSA-trypsin inhibitor were detected (158).

The CL of sulfur-containing compounds is also reported (159). A FIA for thiopronine and its metabolite, 2-mercaptopropionic acid, was developed by utilizing the phenomenon that Ce(IV) CL can be enhanced by quinine (160). In the same manner, rhodamine B was used as an enhancer for the determination of preparations of captopril and hydrochlorothiazide (161,162).

As mentioned above, CL is a versatile tool for sensitive and selective determination of trace amounts of organic and inorganic compounds including biologically important components such as bioactive amines, nucleic acids, sugars, etc. Therefore, it is expected that CL will be utilized to postgenome sciences such as proteome, metabolome, etc.

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