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Review

Development of chemiluminescence reactions in biomedical analysis

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Introduction

In the field of biomedicine, the analysis of organic and inorganic components in biological samples is very important. However, it is often difficult to analyse selectively and sensitively low levels of substances in fluids, especially in blood. Recently, chemiluminescence (CL) systems have proved to be valuable for trace analyses. The quantum yield of CL, i.e. the number of emitted photons divided by the number of reacting molecules, is often very low compared with that of bioluminescence. However, the recent development of sensitive photomultipliers has made it possible to follow very weak luminescence. By using reactions where the quantum yield is of the order of 0.01–1, these analytical methods often attain exceedingly high sensitivities. The advantage of CL over fluorescence detection arises from the elimination of the relatively unstable and background-producing light source. The luminometer or conventional fluorescence detector with the light source off can be operated at very high sensitivity. Thus the CL system is preferable in biomedical analysis where very sensitive methods are required.

The current high level of interest in CL is reflected in the world-wide representation

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and range of papers presented in recent analytical journals, among which is a very specific new journal [1], and symposia [2–9]. Several review articles discuss the history and the phenomenon of CL [10–16]. In this review the application of CL systems to biomedical analysis is discussed.

Chemiluminescence or "cold light" is defined as luminescence derived from a chemical reaction at ordinary temperatures, i.e. the emission of light on return to the ground state from an electronically excited species produced by a chemical reaction without any associated generation of heat.

This phenomenon has been known for a long time. The fascination of man for this physico-chemical appearance is reflected in the writings of antiquity and the early scientific literature [17]. In 1877, Radziszewski [18] found that lophine (2,4,5-triphenylimidazole) emitted green light when it reacted with oxygen in the presence of strong base. This is the first example of CL with an isolated organic compound.

In an impressive study in 1905, Trautz [19] surveyed the known examples of CL, and systematically reported the luminescent properties of the reactions of several hundred organic compounds with various oxidants. In that study, some of the earliest investigations of the spectral distribution of the emitted light were made and the emission of light was attributed to a form of "activated" oxygen. Kautsky *et al.* [20–22] investigated the chemistry of the siloxenes, complex silicon compounds first prepared more than a century ago, which emit a bright light when a suspension of the siloxene in dilute acid is treated with strong oxidizing agents such as permanganate, ceric compounds, or nitric acid, the colour and intensity of the emission varying strikingly with time.

Mallet [23] reported in 1927, that the intensity of light emitted in the reaction of hydrogen peroxide and hypochlorite ion was enhanced when eosin, fluorescein, anthracene, quinine sulphate or aesculin was added to the reaction medium. In the next year, Albrecht [24] described the intense luminescence associated with the alkaline oxidation of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione). This compound and its derivatives have been the most widely investigated CL systems with hundreds of related publications to date.

In 1935, Gleu and Petsch [25] observed an intense green emission when lucigenin (10,10′-dimethyl-9,9′-biacridinium dinitrate) was oxidized in an alkaline medium. The CL system of this compound has also been widely studied. Several strongly luminescent systems have been discovered since then.

Chandross in 1963 [26], and McKeown and Waters in 1964 [27], observed the visible light emitted during the reaction of hydrogen peroxide with oxalyl chloride or certain nitriles. In 1965, Rauhut *et al.* [28] reviewed the oxalyl chloride CL system and showed that oxalyl esters could be used for this system instead of oxalyl chloride. Since then, they have synthesized a number of oxalates including oxamides and established a new strongly luminescing system, namely, the peroxyoxalate CL system. Mohan and Turro [29] described in 1974 a facile and effective CL experiment as a handy pedagogical tool for the demonstration of the peroxyoxalate CL reaction principle. The system comprised a "chemical pump" (e.g. the reaction of an oxalate ester with hydrogen peroxide) and an emitting acceptor (fluorescent compounds or fluorescers). The article contains a description of the synthesis of bis(2,4-dinitrophenyl) oxalate (DNPO) and of bis(2,4,6-trichlorophenyl) oxalate (TCPO). The mechanism of peroxyoxalate CL reaction was proposed by Rauhut *et al.* [15] in 1967, by Catherall *et al.* [30] in 1984 and by Alvarez *et al.* [31] in 1986. Peroxyoxalate reactions have been shown to excite easily oxidizable

fluorescers [32, 33] down to 280 nm, although efficiency decreases markedly in the UV region [34].

Some analytical applications of CL in inorganic analysis are treated in the article of Bark and Wood [35]; Isacsson and Wettermark [36] presented in 1974 an excellent review covering the general field of analytical methods based on the recording of CL. The applications included gas phase analysis, analysis in the solid state and in the liquid phase, and special applications (identification of blood stains in forensic chemistry, the analysis for micro-organisms and the CL of organic compounds induced by ozone).

As discussed earlier, for the assay of very low levels of biological substances, very sensitive and specific methods are desired. One of the most valuable methods for this purpose is radioimmunoassay, developed by Yalow and Berson [37] in 1960. However, a radioimmunoassay has certain disadvantages including health hazards. In 1976, Schroeder *et al.* [38] developed a CL immunoassay as an alternative to radioimmunoassay. By using isoluminol derivatives as fluorescent labels, specific protein bindings were monitored allowing the assay of biotin and thyroxin. A review on CL immunoassay is described by Schroeder *et al.* [39] in 1978, and by Barnard *et al.* [40] in 1985. The peroxyoxalate CL detection system has also been applied to enzyme immunoassay by Arakawa *et al.* [41] in 1982 who developed a sensitive, selective CL enzyme immunoassay of 17 α-hydroxyprogesterone using the glucose oxidase and TCPO-8-anilinonaphthalene-1-sulphonic acid system. The detection limit of the method was 0.5 pg/tube. A review on CL enzyme immunoassay by the peroxyoxalate system was given by Tsuji *et al.* [42] in 1985.

Burdo and Seitz [43] reported, in 1975, the mechanism of the formation of a cobalt–peroxide complex as the important intermediate leading to luminescence in the cobalt catalysis of luminol CL. Metal catalysis of the luminol reaction in chromatographic solvent systems was reported in 1976 by Delumyea and Hartkopf [44], while Yurow and Sass [45] communicated on the structure–CL correlation for various organic compounds with luminol–peroxide. An excellent review on bioluminescence and CL was published by Hastings and Wilson in 1976 [46].

In 1978, Paul [47] focussed attention on newer advances in CL analysis in solution, stressing the high sensitivities that are possible and the use of rather inexpensive equipment. For analysis in solutions the most frequently used CL reaction is the alkaline oxidation of luminol and lucigenin, hydrogen peroxide being the normal oxidant, although sodium hypochlorite, sodium perborate or potassium ferricyanide may also be used. CL reactions involving alkaline oxidation can be used to indicate acid–base or redox titration end-points either by the appearance or the quenching of CL when an excess of titrant is present. The luminol and lucigenin reactions together with the CL oxidation of lophine and siloxene have been most frequently used for CL titrations.

Stieg and Niemann [48] considered flow-cell design for analytical CL. Burguera and Townshend [49] used the CL emission produced by the oxidation of alkylamines by benzoyl peroxide to determine up to *ca* 0.5 μmol ml^{-1} of aliphatic secondary and tertiary amines in chloroform or acetone. Various coiled flow-cells were tested for monitoring the CL produced by the cobalt-catalysed oxidation of luminol by hydrogen peroxide and the fluorescein-sensitized oxidation of sulphide by sodium hypochlorite [50]. Kawasaki *et al.* [51] labelled amines and carboxylic acids with an isoluminol derivative prior to separation by HPLC; post-column reaction with hydrogen peroxide and potassium ferricyanide enabled chemiluminometric detection to be carried out. The post-column reaction with lucigenin of sugars, e.g. glucose [52], heparin [53], steroids, e.g. cortisol

and carboxylic acid *p*-nitrophenacyl esters [54] allowed sensitive detection of these analytes.

Peroxyoxalate CL is subject to catalysis by weak bases (e.g. amines) and inhibition by organic acids. Sherman *et al.* [55] used the TCPO system to determine hydrogen peroxide and aromatic hydrocarbon fluorescers in a static system; when metal chelates are employed as fluorescers, trace metal analysis can be performed.

A unique feature of the peroxyoxalate CL for the sensitive detection of a fluorescer is that the analyte is excited many times generating one photon each time by a large amount of the active intermediate produced in the first step of the CL reaction with aryl oxalate and hydrogen peroxide. Thus, the multiple photons for one analyte are to be determined by a photomultiplier. This feature contrasts with the other CL reactions such as luminol or lucigenin CL, in which at most, one photon for each molecule of the analyte can be generated. However, the fluorescer should be purified before excitation by the peroxyoxalate CL reaction, otherwise the contaminated fluorescers are excited in the same manner and interfere with the detection of the analyte. In this sense, in 1977, Curtis and Seitz [56, 57] applied the peroxyoxalate CL reaction to the detection of fluorescers separated by thin-layer chromatography (TLC). Dansyl derivatives separated by TLC could be detected by successive spraying with solutions of TCPO and hydrogen peroxide in dioxane. The suggested method is comparable to conventional fluorescence detection, and has the advantages that it does not require excitation radiation and can be used to excite the plate uniformly. Also, the system is particularly well suited for photographic detection and can be used in the absence of any power requirements.

Extensive research on the use of aryl oxalates for CL detection of biomedically important substances in high-performance liquid chromatography (HPLC) has been carried out since 1980 by Imai's group from Tokyo. This team determined fluorescers or fluorophor-labelled compounds by HPLC with CL detection [58, 59], using TCPO in ethyl acetate, and hydrogen peroxide in acetone, mixed with the column eluate using separate pumps and a suitable mixing vessel. Dansyl amino acids (alanine, glutamic acid, methionine and norleucine) were separated on a μ-Bondapak C18 column and fmol detection limits could be reached. The experiments in static systems demonstrated that the CL intensity decays apparently according to a first-order rate equation and so the measurements have to be carried out as soon as possible. In fact, the authors state that care should be taken during CL detection in TLC experiments. In flow systems, on the other hand, reproducible peak heights that are proportional to the concentration of the fluorescers are obtained since the detector, which is a fluorescence spectrophotometer with the light source switched off, measures the CL intensity during a fixed stage of the reaction. An analogous TCPO–hydrogen peroxide system was used for the determination of fluorescamine-labelled catecholamines in urine [60], providing a 25 fmol detection limit, which is about 20 times lower than that of a conventional fluorescence detection system, which inherently produces higher background levels because of stray radiation.

Several aryl oxalates were evaluated for the hydrogen peroxide-induced CL detection of various fluorescers for use in HPLC post-column reactors [61]. In 1986, six oxalate esters of 2-nitro-4-alkoxycarbonylphenol and 2-alkoxycarbonyl-4-nitrophenol were synthesized for use in the peroxyoxalate CL reactions [62].

The same group reported in 1986 a sensitive and selective HPLC method followed by CL detection utilizing immobilized enzymes for the simultaneous determination of acetylcholine and choline [63]. Both compounds were separated on a reversed-phase column, passed through an immobilized enzyme column (acetylcholine esterase and

choline oxidase), and converted to hydrogen peroxide, which was subsequently detected by the peroxyoxalate CL reaction. The linear determination ranges were from 10 pmol to 10 nmol. The detection limit for both cholines was 1 pmol. A review on CL detection systems for HPLC was given by Imai in 1986 [64].

The use of oxalate esters for CL detection in HPLC is continuously growing. Poulsen *et al.* [65], for example, investigated the combination of solid-state peroxyoxalate CL detection and post-column chemical reaction systems in liquid chromatography, while De Jong *et al.* [66] developed a low dispersion CL detection system for narrow-bore liquid chromatography. It is clear that many more developments are to be expected in the near future from CL systems applied to the chromatographic determination of several biomedically important fluorescing molecules, native fluorescers or fluorescers generated after suitable labelling of the parent molecule. In this respect, the development of effective labelling reagents for the peroxyoxalate CL reaction will be required.

In the future, a combined method of CL reactions affording high detectability, with HPLC affording high selectivity, will certainly help the development of research in the areas where ultratrace analysis is of great importance.

For this, a greater effort will be required to search for a sensitive, but not necessarily selective, CL reaction for the analyte substances.

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