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Study on Some Factors Affecting the Precision of Luminescence Analyses

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The effects of the shape of the reaction vessel, the sample volume and the volume and speed of the reagent injection on the reproducibility of the chemiluminescence (CL) analyses were investigated with a laboratory-built automated injection device and a computer system. The suitable selections of the vessel diameter as well as the injection volume are shown to be necessary for the precise measurements of samples of given volume. Under the optimal mixing conditions, the choice of signal processings such as peak height measurements or fixed-time integration did not affect the reproducibility and any of the methods used could give accurate CL values. These principles for the precise measurement represent useful guides for luminometer designing and experiments for rapid, precise analyses.

Keywords——luminescence; chemiluminescence; automated injection; computer analysis; mixing mode

Introduction

Chemi- and bioluminescence in analytical chemistry have obvious potential advantages such as superior sensitivity, safety and controllable emission rate, and have been studied widely. Chemiluminescent assays of hydrogen peroxide were estimated to be some 100-fold more sensitive than the corresponding spectrophotometric assays.[1-3] The luminescent methodology, however, has the disadvantages of poor reproducibility and long observation times, although not all the reactions are slow.

Since some assays involve reactions taking place very quickly, the shape and size of a reaction vessel together with the mode of addition of reactants are important for the rapid, precise measurement. In the facile, rapid measurement, chemiluminescence (CL) reactions are often initiated by the injection of an oxidant solution into a small vessel containing a luminescent material and a catalyst, and measured with a commercially available analog recorder; the injection procedure itself causes the mixing of the oxidant and the luminescent solution. However, the reproducibility of the measurements in this system has not been completely satisfactory. For example, the peak light intensity of 15 individual reaction mixtures in a system with manual injection had a coefficient of variation (CV) of 18%.[4,5] A pneumatically-driven automated injection system was developed by Schroeder *et al.*,[6] but it gave only a slight improvement of the precision (CV = 4—9%, $n = 12$). On the other hand, flow injection systems have been useful; one of them provided the coefficient of about 2% for 18 individual reactions.[4] Those systems, however, are not suitable for physicochemical studies of reaction mechanisms or solid-phase immunoassays, since they have long delay times and no easily exchangeable optical parts or vessels for the observation.

The CL behavior on the mixing resulting from the injection is crucially affected by factors such as the shape of the vessel, the sample volume, the injection volume and speed, the

viscosity of the sample solution, *etc.* These factors may be called here a mixing mode or mixing style. An adequate setup of the mixing mode is expected to provide the superior precision of the luminescent measurements.[7] Systematic study of the mixing style, however, requires an automated injection device for a constant mixing manner and a computer system for rapid, precise data acquisition and processings. Such a system was developed in our laboratory.[8] This instrument showed that smoothing by Fourier transform was useful for peak-height detection from raw noisy data, which may be considered the simplest and the fastest, and that only 0.5 s was needed for the measurement of a CL reaction that continued for 40 s.[9] This methodology of rapidity, however, could not be practical in clinical laboratories without a mixing mode that gives the superior reproducibility.

With the luminescence detection system described above, we examined the effects of the mixing mode on the reproducibility of the CL measurements and on the signal processings for the CL analysis. This paper describes some requirements for satisfactory precision in the luminescent measurements. Unfortunately, many factors that affect the reproducibility were not studied here, *e.g.* the viscosity of the sample solution, temperature, *etc.*, but the results described here will be a useful guide for a rapid, precise measurement and luminometer designing.

Experimental

Materials——Luminol and hemin were purchased from Tokyo Kasei, and, microperoxidase (M-6756) from Sigma Chemicals; these were used without further purification. All other reagents were of analytical grade and not further purified. Luminol stock solution was prepared in a glass vial at *ca.* 1 mM in 0.1 M Na_2CO_3–$NaHCO_3$ buffer, pH 10.5, and kept at 4 °C; hemin stock solution was made at *ca.* 0.3 mM in 0.1 N NaOH.[5] The stock solutions were diluted to desired concentrations with 50 mM NaOH. Commercially available 30% H_2O_2 (Santoku Chemical Industries) was diluted to 0.03% with degassed distilled water and its injection volume was 20 or 60 μl. Reaction mixtures consisted of equal volumes of hemin solution (3×10^{-5} M) and luminol solution (10^{-8} M). The preparations of microperoxidase stock solution and its reaction mixture were described previously.[5,8,9] Since the fast emission of the hemin system makes the measurement rapid and easy, hemin was used as a catalyst in most stage of

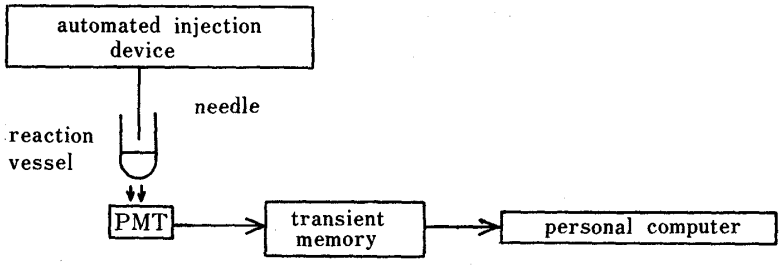

Fig. 1. Block Diagram of Total Luminescence Analyzing System
PMT represents photomultiplier tube.

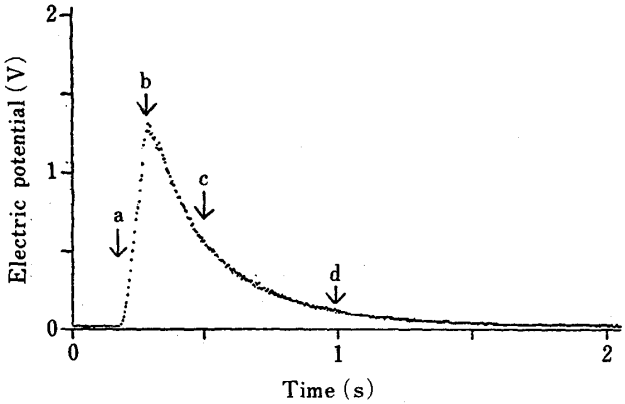

Fig. 2. Printer Output for Chemiluminescence Obtained upon the Oxidation of Luminol Catalyzed by Hemin (Average Data of 5 Experiments)

A small letter a shows the initiation of the CL reaction (the H_2O_2 addition); b, a light peak; c and d, intervals over which the data were analyzed by the computer to give the LE value (see "Data Analysis" in Experimental). The experiment was carried out: the reaction mixture, 300 μl (see "Materials" in Experimental); the injection volume, 60 μl; the needle height from the surface of the sample solution, 5 mm; the inside diameter of the reaction vessel, 8 mm; the needle diameter, 1/3 mm.

these experiments, and the methods were later applied to the slow CL of the microperoxidase system. Temperature was maintained at 23 to 25 °C.

Apparatus——Measurement of light emission was carried out with a pneumatically-driven injection device and a computer system which were developed in our laboratory[8] (see Fig. 1). Hydrogen peroxide solution of constant volume was added to the solution of luminol and a catalyst in the reaction vessel by the automated injection device. The injection unit was driven by a solenoid valve and an air cylinder that were powered by compressed nitrogen gas. The pressure of the gas can be varied to adjust the velocity of the injection. The device can use the vessels of several inside diameters (5, 6, 8, 10, and 14 mm) and heights of less than 50 mm, the addition volume of 10—150 μl and a widely variable injection point (the distance of the injection needle from the surface of the sample solution); all the vessels used here were round-bottomed and had a height of 50 mm. The light emission was recorded by a transient memory (TMR-120, Kawasaki Electronica) and the digitally recorded reaction curve was analyzed by a personal computer (MZ-2000, Sharp).

Data Analysis——Figure 2 shows the typical data for the chemiluminescence reaction of luminol. The light production rapidly increased after the injection of the peroxide solution (see a in Fig. 2), reached a maximum (b) and decayed ($t_{1/2}=0.21$ s) to background electric potential in 2 s. The maximum light intensity was determined from the highest voltage after the noisy data was removed by a digital filter using Fourier transform (FT) as described by Hayashi *et al.*[9] Fixed-time integration of the output was also carried out and the time was selected arbitrarily. We also analyzed 'total emission' (TE) of the CL phenomenon by dividing the CL data into two sums[8]: [1] early emission (EE) is the sum of the light output in the form of the product of electric potential and time (V × s) over the range between a and c in Fig. 2 (EE is one of the fixed-time integrations.); [2] late emission (LE) is derived from a definite integral of the first -order reaction formula from time c to infinity. The 'total emission' is written: TE = EE + LE. It is equivalent to the total quantity of the emission measured over a sufficient time for the completion of the reaction.[8] These methods of FT and TE can save time and effort for experiments.[8,9]

Results and Discussion

Figure 3 shows the dependence of the reproducibility of the CL measurements on the sample volume in the reaction vessel. Under the conditions of 8 mm inside diameter of the vessel and 60 μl injection volume, the optimal sample volume (CV $\leqq$ 2%, $n=5$) ranged from 200 to 400 μl. In the small sample volume region the sample might be scattered by the strong flow of the oxidant so that the precision becomes poor. On the other hand, at large sample volume (over 600 μl) in comparison with the injection volume, the sample might not be mixed thoroughly with the peroxide solution. We also investigated the effect of the height of the injection needle tip from the surface of the sample on the CL precision, but the precision was

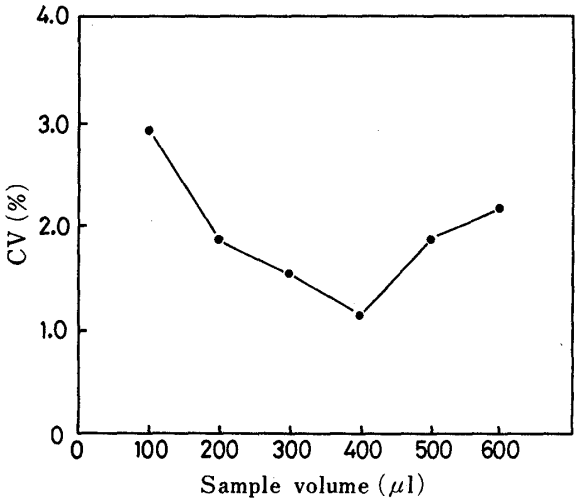

Fig. 3. Effect of Sample Volume on the CL Reproducibility

Each data point represents the CV (%) of the TE's obtained from 50 measurements. The experiment was carried out in the same way as in Fig. 2, except that the sample volume was varied.

Table I. The Optimal Range of Sample Volume

Inside diameter of reaction vessel (mm)	Optimal sample volume (μl)
20 μl injection	
5	200
8	350—400
14	500—700
60 μl injection	
8	200—400
14	600—700

TABLE II. Effect of Signal Processings on Chemiluminescent Reproducibility

	Chemiluminescence intensity (V·s)	CV (%) $n=5$
Rapid reaction[a]		
Integration time (s)		
0—0.4	0.2110	2.51
0.4—1.0	0.2378	1.91
0.4—2.0	0.2754	1.89
0—2.0	0.4854	1.09
Total emission	0.4803	1.10
Peak height	1.377 (V)	1.50
Slow reaction[b]		
Integration time (s)		
0—5	0.1340	3.42
5—20	0.4125	2.35
5—40	0.6645	2.80
0—40	0.7983	2.85
Total emission	0.9003	2.10
Peak height	0.07158 (V)	1.93

a) H_2O_2–hemin system, pH 12.6 ($t_{1/2}=0.21$). *b*) H_2O_2–microperoxidase system, pH 12.6 ($t_{1/2}=12.7$).

not affected in the range of 2-21 mm. The needle was set at the center of the reaction vessel in this experiment.

With other inside diameters of the reaction vessels (5 and 14 mm) and a different injection volume of the peroxide solution (20 μl), the similar experiments were undertaken. Table I shows the optimal ranges of the sample volume in the vessels of different diameters. The CL characteristics of each vessel have a strong resemblance to that of the 8 mm vessel (see Fig. 3). Each vessel has suitable sample volume for satisfactory reproducibility, except that the 5 mm vessel does not have an optimal region for 60 μl injection. This result seems to be due to the relatively large injection volume that may cause excessively vigorous mixing. The vigorous mixing does not necessarily give superior reproducibility, even though the injection might result in rapid, thorough mixing. As a whole, the optimal sample volume increases with increase in the vessel diameter. The results suggest that, depending on the volume of samples, adequate selections of both the diameter of the vessel and the injection volume of the reagent are necessary for the precise assay. The optimal ratio of sample volume to inside diameter of the vessels was 40—50 μl/mm. This ratio will be a useful guide to the selection of suitable vessel for samples of given volume.

Using one of the optimal mixing conditions (inside diameter of vessel, 8 mm; sample volume, 300 μl), we examined the dependence of the CL reproducibility on signal processings. Raw data were manipulated with three methods: fixed-time integration, TE and peak height (see "Data Analysis" in Experimental). Table II shows that each signal processing was effective (CV $\leqq 2\%$, $n=5$) except the short-time integration after the injection (0—0.4 s). This poor reproducibility of the early-time integration might originate from the rapidity at the early stage of the CL reaction immediately after the injection. A similar investigation was conducted by Pazzagli *et al.*[19] but the reproducibility was not satisfactory (CV = 4.8—5.5%, $n=8$), because of their unsuitable mixing modes. Table II also shows that the precision of the measurements is independent of the signal processings, when the experiments are carried out under the optimal mixing conditions, and suggests that the data processings can be selected arbitrarily for the analytical purposes. Peak height determination is the simplest and the most rapid, and should be useful for clinical assays of many samples. Such determination by

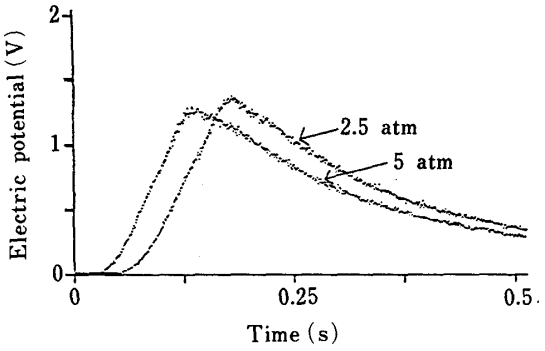

Fig. 4. Superimposed Printer Output Data for Chemiluminescence

Values of 2.5 and 5 atm represent the pressure of the nitrogen gas that powered the drive syringe for the injection. The experimental conditions were the same as that in Fig. 2.

Fourier transform allowed the real-time processing of raw noisy data by a personal computer.[9]

The finding that the signal processings are independent of the reproducibility was applied to the slow reaction, luminol–microperoxidase system (pH 12.6), which is most generally used; *e.g.*, Weerasekera *et al.*[11] and Kim *et al.*[12] utilized this system for solid-phase immunoassays. For the slow reaction ($t_{1/2} = 12.7$ s), the same experiment as that in Table II confirmed the above result (not shown) and led to the conclusion that the rate of the CL reaction did not affect the reproducibility of the measurements under the optimal mixing. The CL reproducibility observed here was superior to that obtained in other injection-type luminescence experiments[4-6] and flow-type experiments.[4] This superiority may be owing to the luminol solution used which is considered to be easy to mix or unsusceptible to the detailed manner of the mixing. Viscous solutions such as concentrated protein solution, however, may alter the optimal mixing mode described above to some extent because of their inferior mixing properties.

Figure 4 shows the effects of the injection speed on the CL reproducibility. The speed can be changed by the pressure of nitrogen gas in our pneumatically-driven automated injection device.[8] The fixed-time integration values obtained at 5 and 2.5 atm drive were not the same: the integration time of 0.25—0.5 s gave the values of 0.128 (5 atm) and 0.151 V × s (2.5 atm). This difference suggests that the poor reproducibility in manual injection systems springs from the scattered injection speed, and that an automated injection device and a rapid data acquisition system are necessary for the rapid, precise measurement. However, the TE values at the different speeds were almost the same. This means that the precision of the luminescence measurement can be recovered by the adoption of the total quantity of the emission at the expense of the rapidity of the measurement. Our luminescence detection system can determine in 0.5 s the peak height values of the slow CL reaction (the microperoxidase system) that continues for more than 40 s and then, with the sufficient mixing mode, does not need the expense.[9]

We have shown that the adequate selections of the inside diameter of the reaction vessel as well as the injection volume of the reagent are necessary for the rapid, precise assay of samples of given volume. On the other hand, the signal processings for the CL measurements can be selected arbitrarily, when the experiment is carried out under the optimal mixing conditions. These guidelines hold for the wide-ranging luminescent reactions. The mixing modes described here will be useful guides to engineers and investigators: luminescent instruments should be able to accommodate several vessels of different diameters; it should also be possible to change the injection volume according to the vessel content, *etc.* We did not examine all the parameters affecting the reproducibility, but the principles of this CL measurement system should be useful in developing other chemi- and bioluminescent assays in the fields of analytical and clinical chemistry.

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