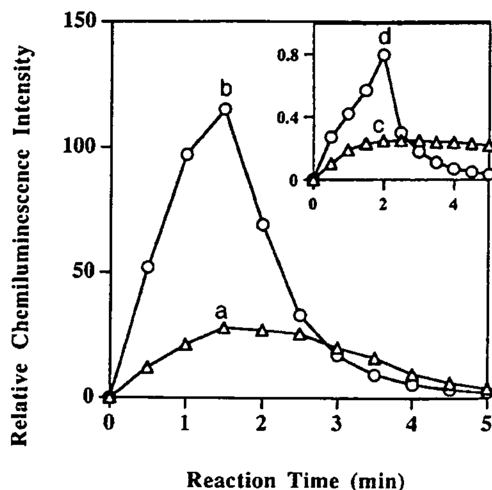

Figure 1. Time course of the kinetic profile of the CL reaction in (a) 5 and (b) 25% acetonitrile and 1 pmol/tube luminol. Inset: (c) 5and (d) 25% acetonitrile alone (0.01 M H_2O_2 and 0.05 M Na_2CO_3).

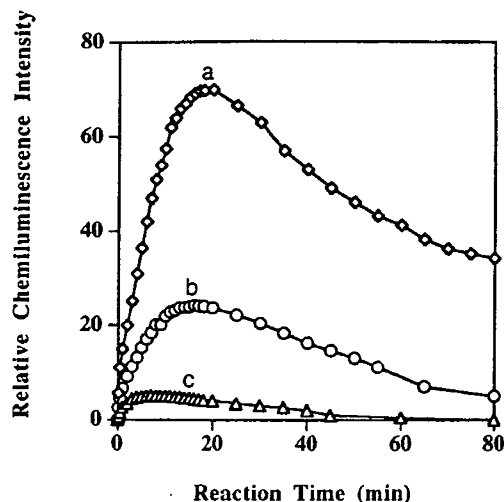

Figure 2. Time course of the kinetic profile of the luminol-enhanced CL reaction; luminol concentration: (a) 100 pmol; (b) 10 pmol; (c) 1 pmol/tube (1% acetonitrile, 0.01 M H_2O_2, and 0.05 M Na_2CO_3).

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

RESULTS AND DISCUSSION

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

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

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

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

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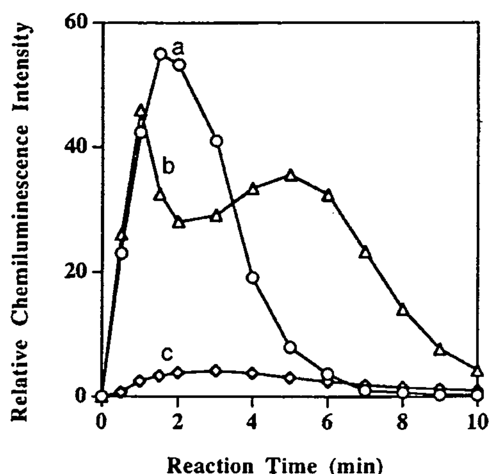


Figure 3. Time course of the kinetic profile of the luminol enhanced CL reaction in 10% different solvent: (a) acetonitrile; (b) propanenitrile; (c) benzonitrile (luminol concentration, 1 pmol/tube).

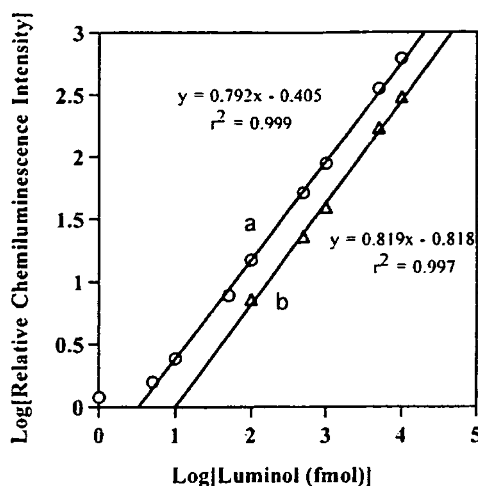
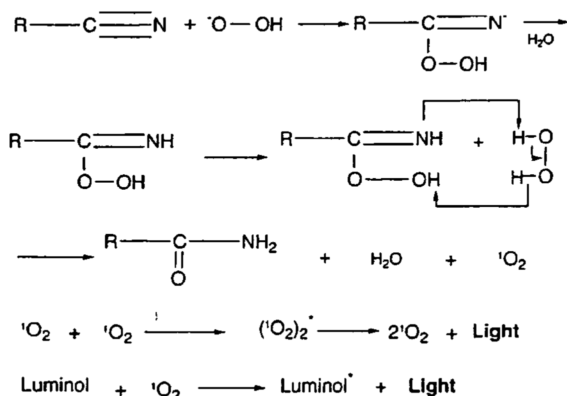


Figure 4. Standard curves of luminol in different concentrations of acetonitrile: (a) 25%; (b) 10%.

Scheme 1



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

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

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

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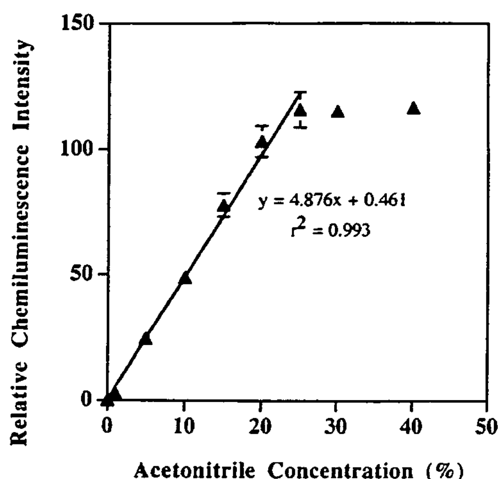

Figure 5. Standard curve of acetonitrile by using the luminol-enhanced CL reaction.

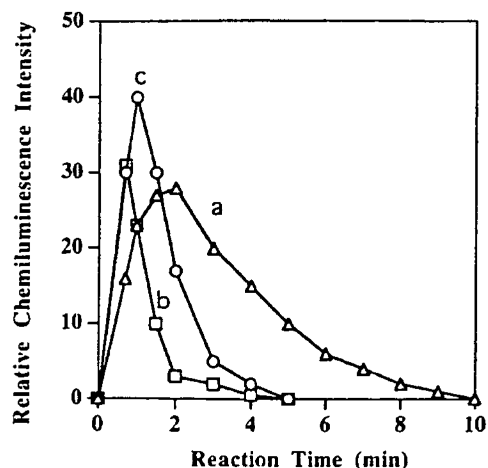

Figure 6. Time course of the kinetic profile of the ClPIC-enhanced CL reaction in (a) pH 9.0, (b) pH 9.6, and (c) pH 11.5 borate buffer (60% acetonitrile, 0.5 nmol/tube ClPIC, and 0.24 M H_2O_2).

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

Furthermore, we found that our recently developed Edman-type fluorescent reagent, ClPIC, could also enhance this new reaction. A typical CL emission profile with the reaction time is shown in Figure 6. The shape of the kinetic profile of this enhanced CL reaction altered as the pH was increased. At pH 9.0, the reaction proceeded more slowly with peak height at 2 min and a decay to background at 10 min. At pH 9.6 and 11.5, the kinetic profiles were more similar consisting of a rapid rise to peak height at 1 min followed by a rapid decay to background by 5 min. CL intensity was maximum at pH 9.6, and the borate buffer was the best one compared with other buffers. Moreover, CL intensity increased with the increase of acetonitrile or H_2O_2

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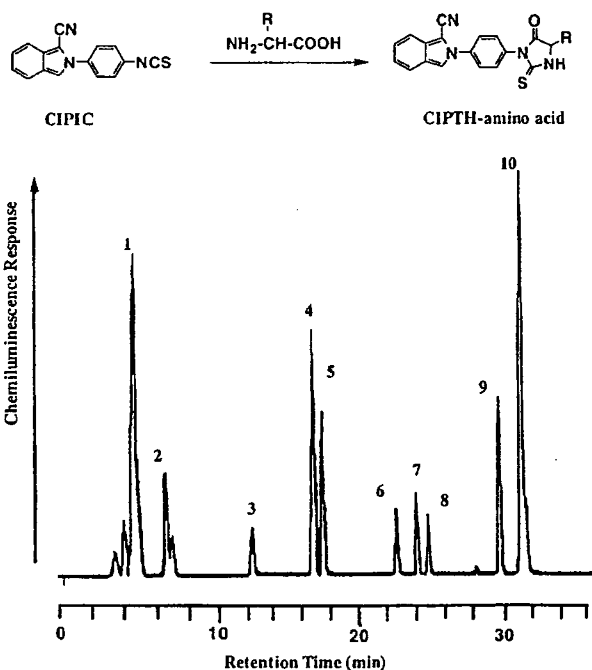

Figure 7. CL chromatogram of six amino acids, Peaks: 1, thiocarbomoyl amino acid residue; 2, α-NH_2-ClPTH-lysine; 3, ClPTH-threonine; 4, byproduct from ClPIC; 5, ClPTH-alanine; 6, ClPTH-proline; 7, ClPTH-isoleucine; 8, ClPTH-phenylalanine; 9, ϵ-NH_2-ClPTH-lysine; 10, byproduct from ClPIC (3.5 nmol for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

concentration. As described previously, ClPIC could react with amino acids with a high efficiency.[10] Thus, as a fourth application, we developed a new amino acid detection method based on ClPIC-enhanced CL reaction between aqueous H_2O_2 and acetonitrile. It is well known that the separation technique plays a key role for the detection of different analytes in complex matrixes. High-performance liquid chromatography (HPLC) has currently become dominant as a potential tool for the separation and quantification of a wide range of analytes in different fields.[31–33]

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

CONCLUSIONS

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

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ORIGINAL PAPER

**Choiwan Lau · Jianzhong Lu · Takashi Yamaguchi
Masaaki Kai**

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

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

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

Introduction

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

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

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

C. Lau · J. Lu · T. Yamaguchi · M. Kai (✉)
School of Pharmaceutical Sciences, Nagasaki University,
1–14 Bunkyo-Machi, Nagasaki 852–8521, Japan
e-mail: ms-kai@net.nagasaki-u.ac.jp

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

Experimental

Chemicals

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

Apparatus

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

Batch CL procedures

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

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

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

Antibody CL imaging on the microplate

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

CL imaging of labeled DNA on a nylon membrane

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

Results and discussion

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

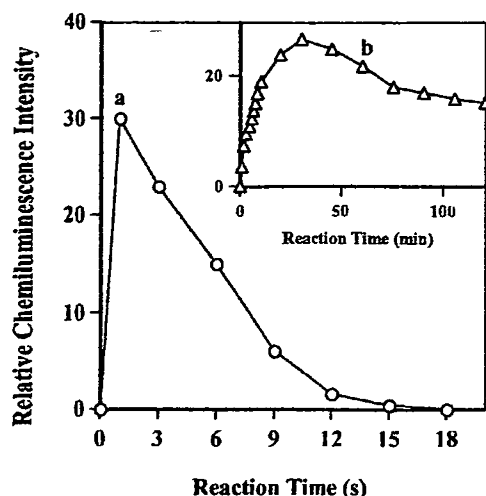

Fig. 1 Time course of the kinetic profile of the CL reaction of 100 pmol/tube luminol in 50 mmol L^{-1} Na_2CO_3 with (*a*) 22 mmol L^{-1} KCN and 1 mmol L^{-1} H_2O_2 and (*b*) 0.12% acetonitrile and 10 mmol L^{-1} H_2O_2

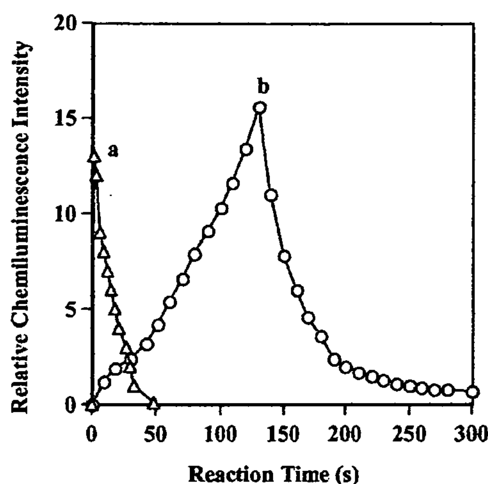

Fig. 2 Time course of the kinetic profile of the CL reaction of 1 nmol/tube fluorescein with (*a*) 22 mmol L^{-1} KCN and (*b*) 10% acetonitrile in the presence of 50 mmol L^{-1} Na_2CO_3 and 20 mmol L^{-1} H_2O_2

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

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

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

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

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

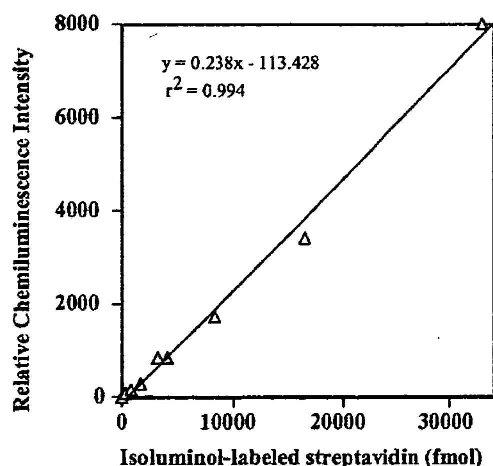

Fig. 3 Calibration curve for isoluminol-labeled strepatavidin obtained by microplate CL imaging with an exposure time of 2 min

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

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

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

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

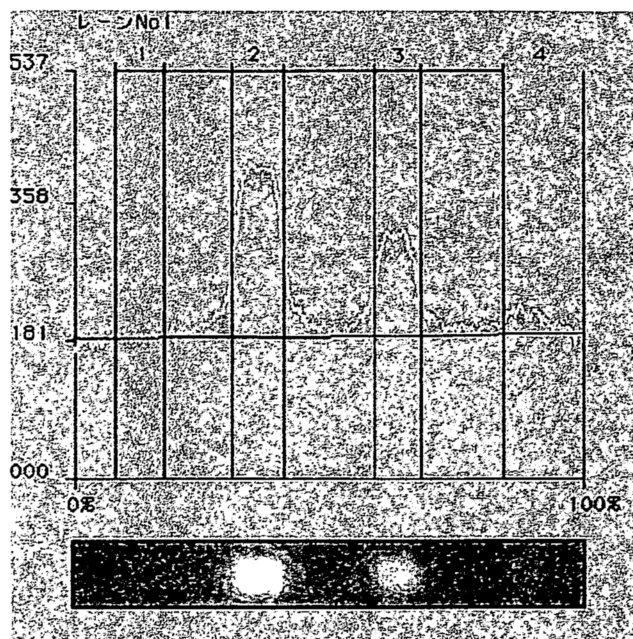

Fig. 5 CL images of biotinylated IgG on the microplate: *1*, blank; *2*, 1000-fold; *3*, 5000-fold; *4*, 10,000-fold dilution

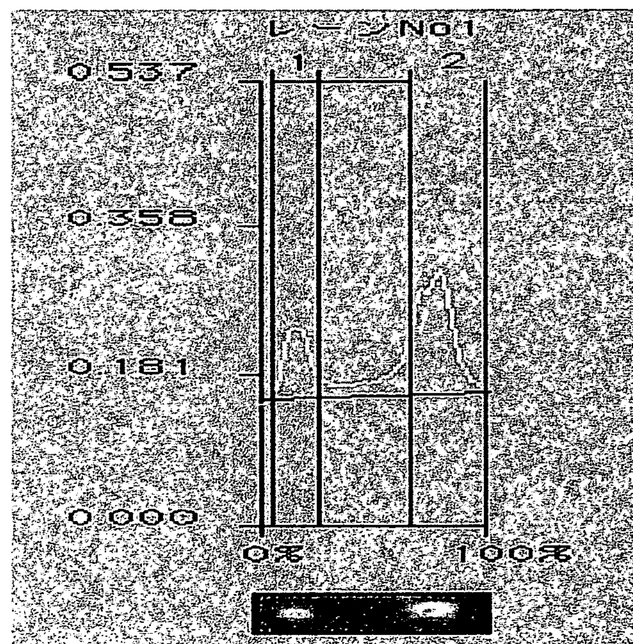

Fig. 6 CL images of nylon membrane with spotted biotinylated DNA: *1*, 400 ng; *2*, 800 ng

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

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

Conclusions

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

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