Determination of total bilirubin in human serum by chemiluminescence from the reaction of bilirubin and peroxynitrite

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Abstract

Peroxynitrous acid (ONOOH) was produced by the on-line reaction of acidified hydrogen peroxide with nitrite in a flow system, and peroxynitrite (ONOO⁻) was generated from ONOOH in NaOH solution. A weak chemiluminescent (CL) emission was observed due to the production of singlet oxygen (¹O₂) during the decomposition of ONOO⁻. Bilirubin and its conjugate were found to enhance the CL emission of ¹O₂ in a suitable micellar medium. For the first time, the feasibility of employing the present CL system for the sensitive and selective determination of total bilirubin contents in human serum was demonstrated and the results were compared with certified values. The present method showed a great improvement on overcoming bis(2,4,6-trichlorophenyl)oxalate CL highly insolubility in aqueous solution and exhibiting higher tolerance towards interferences than redox reaction of bilirubin with various oxidizing agents such as sodium hypochlorite and iodine. The recoveries of bilirubin were found to fall in the range between 95 and 108%. The detection limits (S/N = 3) for bilirubin and its conjugate were determined to be 10 and 8 ng ml⁻¹, respectively. The relative standard deviations (R.S.D.) for the consecutive CL detection of a series of bilirubin (30 μg l⁻¹) and bilirubin ditaurite (25 μg l⁻¹) were 3.2 and 2.9% (*n* = 11), respectively.

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1. Introduction

It is well known that bilirubin is a metabolic breakdown product of blood heme with great biological and diagnostic values. In sera of healthy individuals, bilirubin exists almost completely (more than 95%) in the unconjugated form and the total bilirubin concentrations in blood typically fall in the range between 3.5 and 10 mg l⁻¹ [1]. Abnormal bilirubin concentrations found in human serum or plasma usually signify the presence of a variety of diseases with liver dysfunctions, ranging from neonatal jaundice to infectious hepatitis [2]. Measurement of bilirubin is important for the diagnosis of these diseases. In the clinical laboratories, the most widely used approach for the determination of bilirubin is based on absorption measurements after diazotization of bilirubin in the presence of an accelerator such as alco-

hol or caffeine [3]. However, these absorption methods are time-consuming and exhibit low sensitivity.

In recent years, chemiluminescence (CL) has shown good potential as an attractive technique for routine usage in clinical laboratories due to its outstanding advantages in terms of limits of detection, instrumental simplicity, speed of analysis, reproducibility, and linear dynamic range [4]. To the best of our knowledge, there are only two reports on CL determination for bilirubin [5,6]. Huie and co-workers [5] were the first to demonstrate the feasibility of detecting CL emission from bilirubin in organic solvents, based on the energy transfer from the reaction intermediate(s) of the peroxyoxalate CL reaction to bilirubin. A major limitation of this approach is that peroxyoxalate CL reagents are highly insoluble in aqueous solutions and the CL efficiency can be severely quenched in the presence of water [7]. To overcome these problems, Calokerinos and co-workers [6] described a CL method for bilirubin determination in aqueous media based on the redox reaction of bilirubin with various oxidizing agents, such as sodium hypochlorite and *N*-bromosuccinimide, but which suffered from the low selectivity due to serious interference

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of albumin by enhancing the emission. Therefore, it was not applied for the determination for bilirubin in real samples.

Micelles are dynamic aggregates of amphiphilic molecules and possess a hydrophilic surface and hydrophobic core. In the micellar media, CL intensity is enhanced by light emitting species interacting electrostatically with and binding to micelles, resulting in accumulation of these species in the micellar phase and increase of reaction rate [8,9]. In this study, the effects of three different types of micelles (cationic, anionic, and nonionic) on the CL intensity were evaluated.

Peroxynitrite (ONOO^-) is a strong oxidant and capable of oxidizing a variety of biomolecules [10–12]. ONOO^- was generated from peroxynitrous acid (ONOOH) in NaOH solution, and ONOOH was produced as a result of the reaction of nitrite with acidified hydrogen peroxide [13]. In our previous work [14], a flow injection CL method for the determination of nitrite was reported due to the decomposition of ONOO^- into NO^- and singlet oxygen ($^1\text{O}_2$) (the proposed CL emitter) in the presence of a suitable surfactant (at concentration above its CMC) and a sensitizer (uranine). In the present work, bilirubin and its conjugate were also found to enhance remarkably the CL emission generated from the nitrite–hydrogen peroxide reaction in a suitable micellar solution and was utilized for the determination of total bilirubin contents in human serum. To demonstrate the usefulness of the present CL method for the determination of bilirubin, the selectivity, recovery, precision, and linear dynamic range were examined. The proposed method was developed for the direct determination of total bilirubin contents in human serum and the results were compared with certified values.

2. Experimental

2.1. Reagents

Bilirubin (unconjugated bilirubin IX), bilirubin ditaurite (bilirubin conjugate) and biliverdin (bilirubin oxidized production) were obtained from Porphyrin Products (Logan, UT, USA). Stock solutions (100 mg l^{-1}) of these three types of bilirubin were prepared by dissolving each standard in 1.0 ml of NaOH solution (0.1 mol l^{-1}) and then diluting with ultrapure deionized water (Millipore, Barnstead, CA, USA). A 1.0 mol l^{-1} nitrite stock solution was prepared by dissolving 6.9 g of pre-dried NaNO_2 (Beijing Chemical Reagent Company, Beijing, China) in 100 ml of water. A small amount of sodium hydroxide was added to the nitrite stock solution to prevent its decomposition and 1.0 ml of chloroform was also added to inhibit bacterial growth. The working hydrogen peroxide solution was freshly prepared by volumetric dilution of 30% H_2O_2 with water. Surfactants, such as cetyltrimethylammonium bromide (CTAB), Triton X-100 and Triton X-114 were obtained from Acros (Geel, Belgium).

Fig. 1. Schematic diagram of the flow injection chemiluminescence system. P₁ and P₂, peristaltic pumps; S, NaOH/CTAB/bilirubin; KR, knotted reactor (10 cm); F, flow cell; W, waste.

2.2. Apparatus

The schematic diagram of the flow system is shown in Fig. 1. It consists of two peristaltic pumps (SJ-1211, Atto, Tokyo, Japan), a CL detector (Lumiflow LF-800, NITI-ON, Funabashi, Japan) and a 50- μl loop injector placed close to the luminometer. Black Teflon tube (1-mm i.d.) was used for the flow lines. The peak height of the signal recorded was measured as CL intensity. For measuring the CL spectrum of the reaction of bilirubin with ONOO^- , the data points from 500 to 600 nm were obtained by measuring the CL signal (peak area) of a new sample solution for each data point (a PTI QM1 luminescence spectrofluorometer, Photon Technology International, Canada). The excitation lamp was off and the emission slit width was opened maximally to 20 nm during the CL spectrum recording.

2.3. Procedures

As shown in Fig. 1, a 50- μl mixing solution of NaOH/surfactant/bilirubin was injected into the carrier stream (water) and mixed with ONOOH , which was produced on-line by the reaction between nitrite and acidified hydrogen peroxide in a spiral flow CL cell. This flow cell was mounted directly in front of the photomultiplier tube of the luminometer, and the CL signals generated from the CL cell were recorded with a Shimadzu U-125 MN recorder (Shimadzu, Kyoto, Japan). A 10 cm knotted reactor was used to enhance the mixing of nitrite and $\text{H}_2\text{O}_2/\text{HCl}$ solution.

2.4. Serum samples pretreatment

Human serum samples were obtained from Bio-Rad Laboratories, Diagnostics Group, Irvine, CA. These samples were prepared from human serum with enzymes, nonprotein constituents, nonhuman protein, and bacteriostatic agents added. Also, these samples were kept frozen and stored in dark before the experiments. Before direct injection of the samples, a dilution with the NaOH/CTAB mixing solution was made for each sample, so that the final analyte concentrations were within the calibration range $13\text{--}110 \text{ }\mu\text{g l}^{-1}$.

3. Results and discussion

3.1. Possible mechanism of the present CL system

Many investigations [15,16] have discussed the decomposition mechanism of ONOO^- and confirmed that the decomposition of ONOO^- generates $^1\text{O}_2$, an excited state of molecular oxygen. Two quenchers of $^1\text{O}_2$, 1,4-diazabicyclo [2,2,2,2]octane (DABCO), and NaN_3 [17], were used in the present experiment. The results showed the CL intensity of bilirubin with ONOO^- involved on-line reaction of nitrite with acidified hydrogen peroxide was decreased in the presence of DABCO or NaN_3 . Also, the CL emission decreased with an increase of DABCO or NaN_3 concentration. This phenomenon provided strong evidence that $^1\text{O}_2$ did play a primary role in chemiexcitation.

Bilirubin can be oxidized to biliverdin by some strong oxidants, such as sodium hypochlorite [6] and iodine [18]. ONOO^- is also a powerful oxidant and capable of oxidizing a variety of biomolecules [10–12]. In the present work, bilirubin, bilirubin ditaurite (bilirubin conjugate), and biliverdin (bilirubin oxidized production) were found to enhance the CL emission generated from the nitrite–hydrogen peroxide reaction. These results may be due to the fact that bilirubin is oxidized to biliverdin by ONOO^- , and biliverdin enhances the CL emission of $^1\text{O}_2$ by the energy transfer from $^1\text{O}_2$ to biliverdin.

Fig. 2 showed the CL spectrum from the reaction of ONOO^- with bilirubin. It is important to note that the CL spectrum of CL is similar to the fluorescence spectra of bilirubin reported in the literature [19,20], suggesting that the CL emission may be a result of a radiative transition of electrons from the first singlet excited electronic state to the

ground state of bilirubin. It can be seen that the CL maximum wavelength of bilirubin is located at ca. 530 nm, which is within the 505–545 nm range reported for the fluorescence maxima of bilirubin bound to micelles. The variation in the fluorescence maximum is due to the different environments in the conformation or ionization of bilirubin [21].

3.2. CL method

In this present work, the CL signal was produced by the decomposition of ONOO^- into NO^- and singlet oxygen (the proposed CL emitter), which had been discussed in our previous work [14]. ONOO^- was generated from ONOOH in basic solution, and ONOOH was produced as a result of the reaction between nitrite and hydrogen peroxide in the presence of an acidic catalyst. The CL signal was proportional to the concentration of nitrite and its intensity was enhanced significantly in the presence of a suitable surfactant (at concentration above its CMC) and/or a sensitizer. Fluorescent compounds, such as uranine and fluorescein, were found to be effective sensitizers (supposedly due to the efficient transfer of energy from singlet oxygen to the fluorescent compounds) for the determination of nitrite in natural water samples. In the present work, bilirubin and its conjugate were also found to enhance the CL emission generated from the nitrite–hydrogen peroxide reaction.

3.3. Optimal conditions for the FIA–CL system

To establish the optimal conditions for the flow injection analysis of bilirubin, the ratio of the peak height of CL signal to noise (S/N) was measured as a function of the concentrations of HCl, NaOH, nitrite, H_2O_2 , and CTAB.

The formation of ONOOH from the reaction of nitrite and hydrogen peroxide needs the presence of acid as catalyst. Based on our previous work [14], a concentration of 0.1 mol l^{-1} HCl was chosen in further experiments.

The CL intensity was strongly dependent on the concentration of NaOH. In the absence of NaOH, no CL signal was obtained with our CL apparatus. A concentration of 0.12 mol l^{-1} NaOH was operated as one of FIA–CL optimum conditions.

The effects of nitrite and hydrogen peroxide concentrations on the CL intensity were also examined, respectively. The results showed that the optimal concentration of nitrite and hydrogen peroxide were 0.01 and 0.03 mol l^{-1} , respectively.

Micelles have been demonstrated to influence the chemistry and photophysics of molecules by altering microviscosity, local pH, polarity, reaction pathway or rate [9]. Also, micelles as reaction medium can enhance CL intensity. In this work, the effects of three different types of micelles on the aqueous ONOO^- with bilirubin were compared for their ability in yielding the highest CL intensity for the detection of bilirubin. Table 1 showed anionic and nonionic surfactants

Fig. 2. CL Spectrum of the (a) peroxyxynitrite/bilirubin reaction and (b) blank. The blank solution consisted of H_2O_2 , HCl, nitrite, NaOH, and CTAB. The concentrations of H_2O_2 , HCl, nitrite, NaOH, and CTAB were 0.03 , 0.1 , 0.01 , 0.12 , and 0.01 mol l^{-1} , respectively. The flow injection method was used. The flow rates of $\text{H}_2\text{O}_2/\text{HCl}$ and nitrite solution were 2.0 ml min^{-1} . The NaOH/CTAB/bilirubin mixing solutions flow rate was 2.5 ml min^{-1} . The excitation lamp was off and the emission slit width was opened maximally to 20 nm . For the measurement of the CL spectrum of bilirubin, the sample solution consisted of 1.0 mg l^{-1} bilirubin standard solution.

Table 1
Effect of surfactants on the CL signal

Surfactant	Conc. (mmol l ⁻¹)	Relative intensity ^a
None		1.0
Cetyltrimethylammonium bromide (CTAB)	10.0 (0.9) ^b	53.6
Myristyltrimethylammonium bromide (MTAB)	8.0 (3.5)	43.6
Didodecyldimethylammonium bromide (DDDAB)	3.0 (0.18)	1.0
Octadecyltrimethylammonium chloride (OTAC)	6.0	48.2
Ethyltrimethylammonium bromide (EDAB)	8.0	50.1
Dodecyltrimethylammonium bromide (DTAB)	12.0	27.5
Diocetyltrimethylammonium chloride (DODAC)	1.0	1.0
Didodecyldimethylammonium bromide (DODAB)	1.0	1.0
Sodium dodecyl sulfate (SDS)	9.0 (8.0)	1.0
Triton X-100	0.1% (v/v)	1.0
Triton X-114	0.1% (v/v)	1.0
Tween-85	0.1% (v/v)	1.0

^a Normalized with respect to the signal in the absence of surfactant.

^b The values in parentheses are the critical micelle concentration (CMC).

had no effect on the CL intensity, and some cationic surfactants, such as CTAB and EDAB, were found to enhance CL intensity effectively. Especially, CTAB can increase the CL efficiency of the present system about 54-fold and the CL intensity is increased near linearly with an increasing in the CTAB concentration from 0.002 to 0.01 mol l⁻¹ (Fig. 3), but stayed relatively constant when the CTAB concentration exceeded 0.01 mol l⁻¹. Therefore, a concentration of 0.01 mol l⁻¹ CTAB was chosen as the optimum condition on account of the solubility of CTAB in water.

3.4. Analytical performance

Under optimum experimental conditions employed in the present study, the calibration curves were found to be linear from 13 to 110 µg l⁻¹ for bilirubin and 10 to 95 µg l⁻¹ for bilirubin ditaurite, respectively. The detection limits (S/N = 3) for bilirubin and its conjugate were determined to be 10 and 8 ng ml⁻¹, respectively, which is ca. five times lower than those reported for the FIA–CL detection of bilirubin in aqueous media based on redox reactions [6]. The relative standard deviations (R.S.D.) for the consecutive CL detection of a series of bilirubin (30 µg l⁻¹) and bilirubin

ditaurite (25 µg l⁻¹) were 3.2 and 2.9% ($n = 11$), respectively. The regression equations were $Y = 0.4231X + 2.2536$ ($r = 0.9975$) for bilirubin and $Y = 0.3126X + 1.9526$ ($r = 0.9968$) for bilirubin ditaurite, where Y is the relative CL intensity and X is the concentration of bilirubin or its conjugate, respectively.

3.5. Interferences

To demonstrate the selectivity of the developed method for the detection of total bilirubin, the effects of typical commonly present in serum such as albumin, glucose, urea, uric acid, riboflavin, hemoglobin, and protoporphyrin were investigated. The concentrations of these interference species added to 50 µg l⁻¹ bilirubin were representative of the diluted concentrations with the NaOH/CTAB mixing solution according to their normal concentrations usually present in human serum [22]. The results showed that the CL signals of bilirubin with and without the presence of these common interference species were about the same, which clearly demonstrated the high selectivity of the present method for the determination of bilirubin.

3.6. Analysis of real samples

In order to evaluate the applicability and reliability of the proposed methodology, it was applied to the determination of total bilirubin concentration, i.e. bilirubin and its conjugate, in human serum samples. The analytical merits of the present method were evaluated by comparing the total bilirubin contents of certified reference human serum samples obtained between the present method and an established clinical method (modified Malloy–Evelyn) [23]. As shown in Table 2, the results obtained with the two methods were in good agreement for the determination of total bilirubin contents in normal serum samples. Also, the recoveries for bilirubin in spiked serum samples were found to be between 95 and 108%.

Fig. 3. Effect of concentration of CTAB. The concentration of bilirubin was 30 µg l⁻¹. All other conditions as in Fig. 2.

Table 2
Determination of total bilirubin in quality control serum (QCS) samples

QCS sample	Certified ^a (mg l ⁻¹)	Measured ^b (mg l ⁻¹)	Spiked (mg l ⁻¹)	Recovery (%) ^b
1	6.0	6.1 ± 0.1	5	98 ± 1.5
			10	106 ± 2.0
2	6.0	5.8 ± 0.2	5	103 ± 2.5
			10	105 ± 4.0
3	6.0	6.2 ± 0.2	5	95 ± 2.0
			10	108 ± 1.5

^a The certified total bilirubin values were established using a standard clinical method (modified Malloy–Evelyn [23]) as quoted by the manufacturer (Bio-Rad Laboratories, Diagnostic group).

^b The total bilirubin contents were measured using the present method and represented the mean of three measurements ± S.D.

4. Conclusion

The proposed method demonstrated the feasibility of employing the reaction between nitrite and hydrogen peroxide, which is compatible with aqueous samples, for the sensitive and selective determination of total bilirubin concentration in human serum samples. When compared to the CL analytical methods in the literature, which employed organic peroxyoxalate CL and redox reaction of bilirubin with various oxidizing agents, respectively, the present study overcame the high insolubility of the bis(2,4,6-trichlorophenyl) oxalate CL reaction in aqueous solution and exhibited higher tolerance towards interferences than the redox reaction of bilirubin with various oxidizing agents.

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