สำนักงานวิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER III

A NOVEL CHEMILUMINESCENT HOST: 5-((3-(((2-HYDROXY-5-METHYLBENZYL)(METHYL)AMINO)METHYL)-2-HYDROXY-5-METHYLPHENYL) DIAZENYL)-2,3-DIHYDROPHTHALAZINE-1,4-DIONE COMPOUNDS IN FLOW INJECTION SYSTEM

3.1 Abstract

A novel chemiluminescent reagent from *N*,*N*-bis(5-methyl-2-hydroxybenzyl)methylamine and luminol is proposed. The obtained compound forms the complex with Cu(II) ion which is an important catalyst in the chemiluminescence reaction of luminol in the optimal ratio 2:1 as clarified by UV-Vis. The optimum condition for chemiluminescence reaction in flow injection system is flow rate 1 ml/min, [NaOH] 0.5 mM, and [H_2O_2] 20 mM. The using of Cu(II) and Co(II) as catalysts result in the ~8 times higher chemiluminescence intensity than others metal (Ca, Pb, Li, Ni, etc.). The chemiluminescence response of the obtained compound is linearly proportional to the concentration of Cu(II) in the range of 0.5-3.25 mM. The CL reaction of novel compound can performs without adding chelating agent as a function of complexation of novel compound and Cu(II).

3.2 Introduction

Flow injection (FI) is now well established as a powerful sample handling technique for laboratory analysis that is compatible with a wide range of detection systems. In its most basic form, it is a convenient means of presenting samples to the detector in a control and reproducible manner. It can also be used to great effect for the on-line physical and chemical pretreatment of samples, e.g. by the incorporation of solid phase micro-reactors containing immobilized enzymes, ion-exchange resins or absorbents.[1] With specific regard to chemiluminescence (CL) detection FI provides the ability to mix efficiently. A photomultiplier is commonly used as the

detector but solid state devices, scintillation counters and modified spectrophotometers and fluorometers have also been used.[2]

Luminol (*5*-aminophthalylhydrazide), when oxidized by most strong oxidants in a basic aqueous solution gives rise to a characteristic blue luminescence. The most popular oxidant is hydrogen peroxide and the reaction is catalyzed by a variety of metal ions. The luminol reaction, in conjunction with FI, has been used to determine a variety of species. The most common applications have involved the direct determination of the reaction components, i.e. H_2O_2, luminol and various catalysts. Early work concerned the determination of H_2O_2 using simple manifolds with either microperoxidase[3] or Cu(II)[4] as the catalyst. The use of various immobilization procedures for the analysis of H_2O_2 has been reported by Nieman and co-workers, e.g., by attachment of luminol, haemoglobin and horseradish peroxidase onto modified silica particles, via glutaraldehyde coupling, in a single-line FI manifold.[5-7] Several metal ions catalyse the luminol reaction, principally Co(II), Cu(II), Fe(II), Cr(III) and Ti(III).[8] These metals have been determined by FI-CL with nmol/L to sub-nmol/L detection limits. An early example was the detection of 1 pg of Co(II) using a coiled glass flow cell and a modified spectrophotometer.[9] An interesting application of this catalytic effect was exploited to determine a range of metal ions in solution based on the displacement of Cu(II) ions from a strongly acidic ion-exchange column.[10] A relatively development has been the use of FI with CL detection to optimize the postcolumn reaction conditions for ion chromatography in order to simultaneously determine groups of metal ions that catalyze the luminol reaction.[11-13] The production of H_2O_2 from enzyme-mediated reactions has allowed the determination of a wide range of organic species. The most common use of *in situ* H_2O_2 generation is for the determination of glucose by oxidation with soluble or immobilized glucose oxidase.[14] Substrates of other oxidases can be determined in a similar manner, for example, sucrose, maltose, lactose and fructose.[15]

CL reaction of luminol is greatly stimulated by the presence of transition metals, through enhancement of initiation reactions, as well as through metal ion catalysis reactions.[16] Generally, the luminol CL reaction requires the complexation of

metal and chelating agent to maintain the solubility in basic pH. Chelators can alter the rate of metal-catalyzed oxidation by steric effects, variations in redox potentials, and alterations in solubility properties of the metal[17] depending on the concentration of the chelator and on the metal ion. Several studies have been conducted to determine the effects of metal chelators on oxidation,[3, 5, 18] however, the effects are complicated and not fully understood.[19] Most of them result in terms of negative effect by decreasing in the fluorescence intensity. In FI system, chelating agent is needed so the effects of using chelator are neglected. The synthesis of luminol derivatives which has metal-chelating properties is one of the challenging to avoid the using of external chelator and obtain the simple FI system. In previous, our group reported *N,N*-bis(*2*-hydroxybenzyl)alkylamines obtained from a single-time ring opening reaction of benzoxazine with phenol derivatives.[20] Considering the basic structure of *N,N*-bis(*2*-hydroxybenzyl) alkylamine, there are many possibilities to proceed the reaction through aromatic ring and hydroxyl group such as esterification, etherification and diazotization. In 2004, Phongtamrug *et.al* reported that *N,N*-bis(*2*-hydroxybenzyl) alkylamine derivatives showed host-metal complex with Cu(II) in both solid and solution state as confirmed by x-ray crystallography.[21] The conjugating onto luminol leads to novel CL compound with unique properties. For example, the linking of luminol-calixarene gives the water soluble spectroscopic calixarene.[22] Based on this viewpoint, we considered the introduction of luminol onto *N,N*-bis(5-methyl-2-hydroxybenzyl)methylamine by diazotization reaction and expected that this novel host compound might interacts with transition metal such as Cu(II) to keep the solubility without adding the chelating agent. The work focuses on the molecular design and simple synthesis pathway of the luminol-*N,N*-bis(5-methyl-2-hydroxybenzyl)methylamine compounds, host-guest complex with metal ion including the CL properties in the FI system.

3.3 Experimental

Chemicals

Luminol, $NaNO_2$ and $CH_3CO_2Na{\cdot}3H_2O$ were purchased from Fluka, Switzerland. EDTA, $CDCl_3$ and aqueous 30% H_2O_2 were obtained from Merck, Germany. Sodium hydroxide and urea were provided from Carlo Erba, Italy. Copper(II) chloride and cobalt(II) chloride were purchased from Shimakyu's Pure Chemicals, Japan, and Ajax Finechem, Australia, respectively. *N,N*-dimethylformamide (DMF) and 37% HCl were from Labscan, Ireland. All chemicals were used without further purification.

Instruments

Infrared spectra were taken in KBr disks on a Bruker Equinox55/S and recorded in the range 4000–400 cm^{-1} with 32 scans at a resolution of 2 cm^{-1}. UV-Vis absorbance data were obtained on a Perkin Elmer UV-VIS spectrometer Lambda 16. The FI system used was equipped with two peristaltic pumps (Ismatec CA4E, 20 rpm) and an electrically actuated rotary six-port valve (Rheodyne, Model 5301). A three way solenoid switching valve (LEE, Model No LFAA 1201618H) was used to introduce the solution between samples. Valve switching and data acquisition was performed using Flow Control Software (A-Chem Technologies, Melbourne) and was run on a computer. PTFE (i.d. 0.8 mm) tubing was used for knitted reaction coils and all flow tubing. The set up FI manifold was shown in Figure 3.1.

Synthesis

The synthesis of *N,N*-bis(5-methyl-2-hydroxybenzyl)methylamine, **1**, was prepared as reported previously.[20] To a solution of luminol (1.45 g, 8 mmol) dissolved in 20 ml of 0.5 M NaOH, 10 ml of 4 M HCl was added with constant stirring. The freshly precipitated luminol was cooled to 0-5°C and diazotized with 5 ml of $NaNO_2$ (0.56 g, 8 mmol) solution. After half-an-hour stirring, a clear brown solution of diazo salt was obtained. A little urea (0.2 g) was then added and the solution was stirred for a further 10 min. Then, the diazo-salt solution was slowly added to a stirred mixture of $CH_3CO_2Na{\cdot}3H_2O$ (1 g) and **1** (0.85 g, 2 mmol) in 50 ml DMF under ice cooling. The

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red reaction mixture was allowed to couple for 2 h at pH 6-8 in an ice bath and then poured into water (250 ml). The resulting solution was acidified to pH ~1 with HCl, and a large quantity of the dark red precipitate formed was then filtered and washed with water. After drying, the crude product weighed 1.8 g, which was purified twice by dissolving in chloroform (90 ml) and reprecipitating with 4 M HCl (~270 ml), followed by washing with water. The purified product (1.2 g, 51% yield) was obtained as a reddish brown solid, **3**.

Complexation in Solution

DMF solutions of **3** and copper chloride (1.65×10^{-4} M) were made up and the two solutions were mixed in the following ratios: **3**-copper chloride, 1:5, 2:4, 3:3, 4:2, and 5:1, respectively. The mixtures were shaken vigorously for 1min and left for 12 h. UV-Vis absorbance at the maximum peak position was measured and plotted as Job's plot.

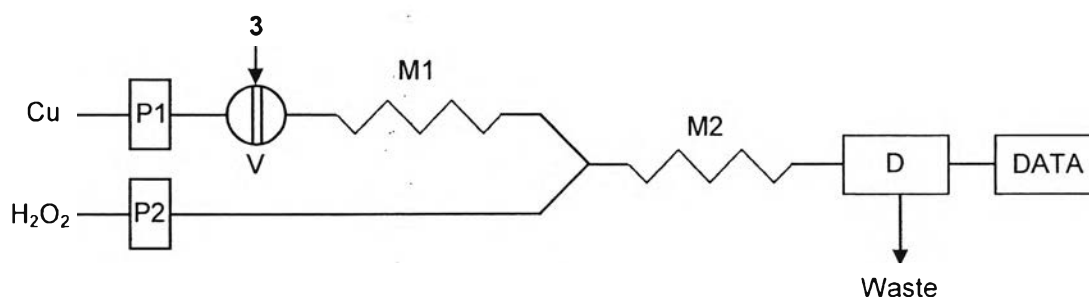
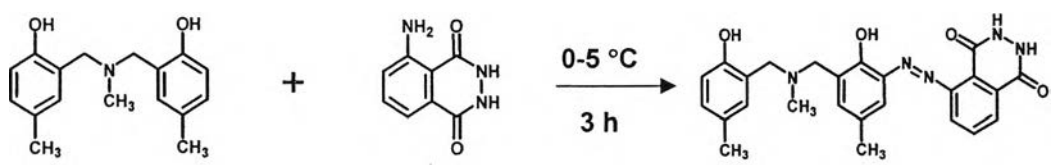


Figure 3.1 FI manifold set up (P1, P2: peristaltic pumps, V: injection valve, M1, M2: reaction coils, D: flow-through photometric detector).

3.4 Results and Discussion

3.4.1 Synthesis and characterization of 3

The preparation of **3** was shown in Scheme 3.1. The result was confirmed by FTIR (Figure 3.2). Compound **1** shows the characteristic peaks of O–H stretching at 3550-3100 cm^{-1} and C-N stretching 1245 cm^{-1} and of luminol at 3050-2800 cm^{-1} (N-H stretching) and 1657 cm^{-1} (2nd aromatic amine). The peak observed at 3271 cm^{-1} for **1** suggested that the hydrogen bond network. After introducing luminol (Figure 3.1C), the peak at 3412 cm^{-1} shows the remaining of hydroxyl group implying the intermolecular H-bond. The peaks at 3350-2880 and 1657 cm^{-1} confirm the luminol group. Moreover, molecular weight could be determined by electrospray TOF-MS (Figure 3.3). Analyses revealed a major peak at m/z = 460.2 corresponding to the molecular weight of **3**.



Scheme 3.1 Synthesis of **3**

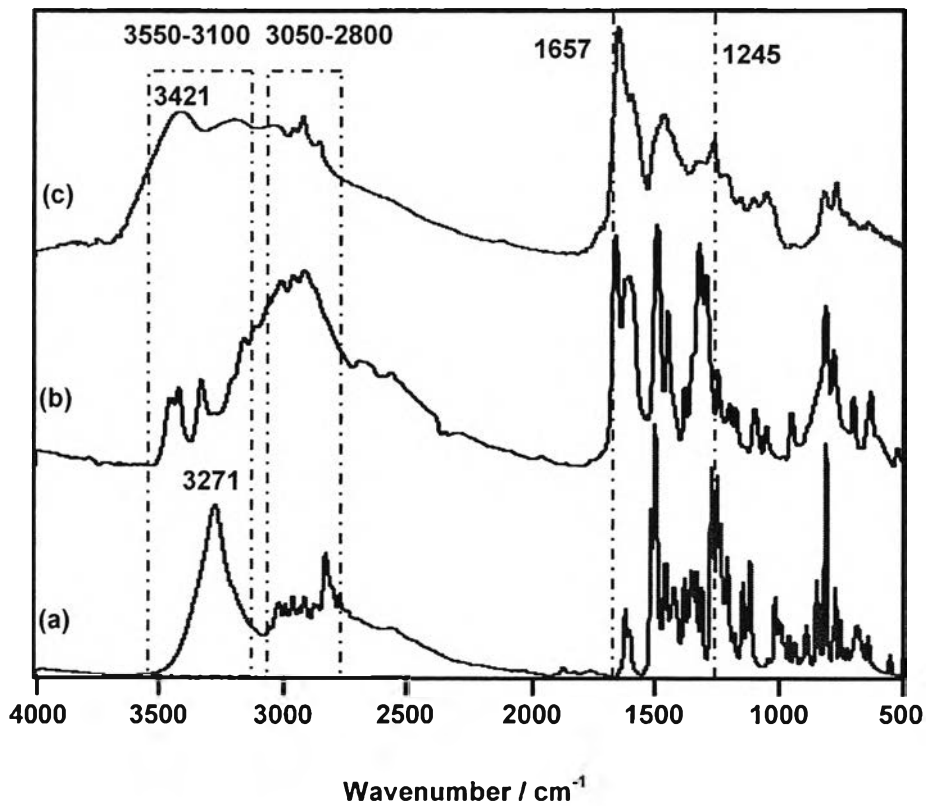


Figure 3.2 FTIR spectra of (a) **1** (b) **2** and (c) **3**.

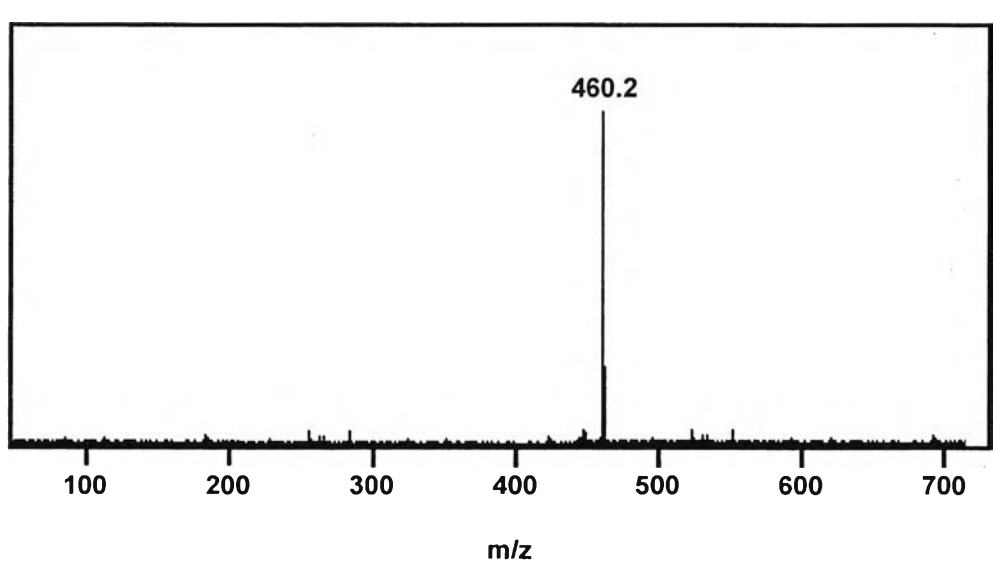


Figure 3.3 ESI-TOF Mass spectrum of **3**.

3.4.2 Host-Guest Complexation of 3 and Cu(II) ion

In order to clarify whether the interaction between **3** and various transition metal ions is formed, UV-Vis spectra were applied to check for the peak shift or the new peak generation. Figure 3.3 shows UV-Vis spectra of the solution **3** with $CuCl_2$ in dimethylsulfoxide for various ratios. Compound **3** gives a maximum peak at 330 nm whereas $CuCl_2$ gives a peak at 289 nm. After mixing, a new peak at 528 nm is observed implying the interaction of **3** with $CuCl_2$. Figure 3.4B is re-plotted from Figure 3.4A to represent the optimal ratio between **3** and $CuCl_2$. The job's plot obtained from the new peak at 528 nm indicates the optimal ratio of **3** and $CuCl_2$ is 2:1. Moreover, we extended our work to clarify the coordinate ion effect by changing from $CuCl_2$ to $CuSO_4$ and $C_4H_6CuO_4$. The optimal interaction was found in the similar ratio. In order to study the interaction of **3** with other transition metal ions, $CoCl_2$ and $C_6FeK_3N_6$ which are the important catalysts in CL reaction of luminol were used. The disappearance of new peak or peak shift implied that no interaction between **3**, $CoCl_2$ and $C_6FeK_3N_6$.

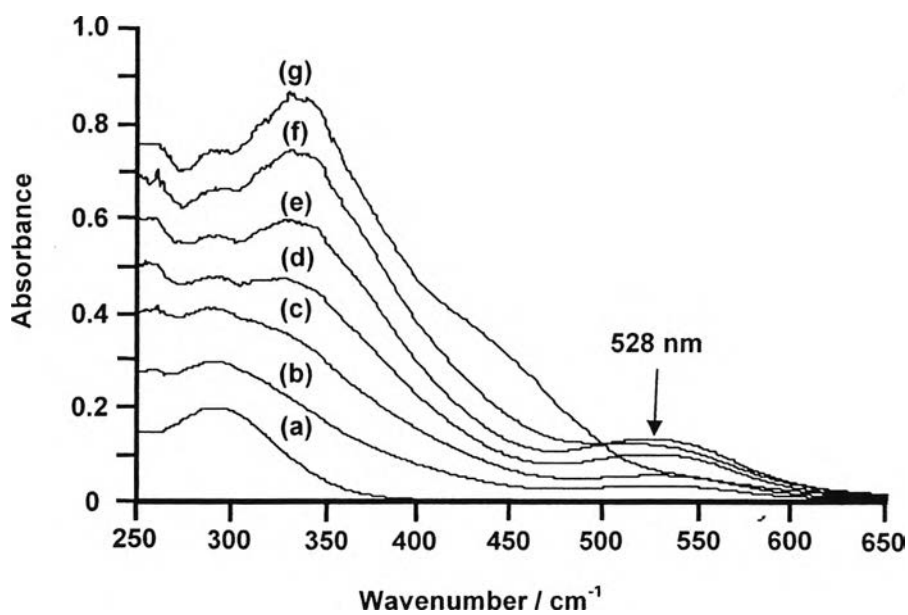


Figure 3.4A UV-Vis spectra of **3**-$CuCl_2$ in DMSO at various volumetric ratios; a) 0:6, b) 1:5, c) 2:4, d) 3:3, e) 4:2, f) 5:1, and g) 6:0.

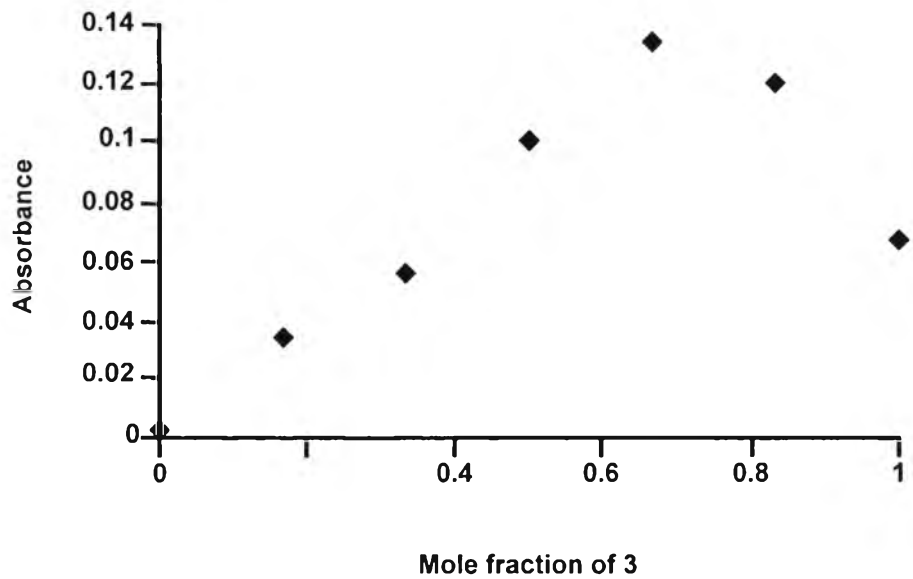


Figure 3.4B Job's plot as a function of mole fraction of **3** at 528 nm.

3.4.3 Chemiluminescence Properties

Two criteria were applied in determining the optimum conditions for the flow injection manifold, i.e., (i) the highest sensitivity and (ii) the shortest practicable analysis time. However, because the method also involves the formation of hydroxide flocculation, there are some blockage problems in the flow lines. This needs the specific condition of reagents, injection volume and flow rates.

The CL properties of **3** were observed by using the flow injection analysis (FIA). The condition for optimum detection was found to be 2 mM of **3** in 0.1M NaOH into 10^{-2} mM of Cu and 50 mM of H_2O_2 with the flow rate 0.5 ml/min. The CL spectra were recorded as a function of voltage.

3.4.3.1 Effect of Chemical and Instrument Conditions

The CL intensities depend on the types of metal ion as catalysts, flow rates and the concentration of NaOH, hydrogen peroxide, the complexation of **3** and the type of metal ion. The effects of the reagents on the CL reaction were examined. The concentrations of the reagents were varied to determine the maximum intensity.

3.4.3.1.1 Effect of Flow Rate

Flow rate is a key factor for this flow sensor, which depends on the analytical speed efficiency and the sensitivity of the sensor. The influence of flow rate on the CL detection system was investigated in the range of 0.1 - 2 ml/min as shown in Figure 3.5. As reported previously, the lower the flow rates, could the longer the contact times between sample and catalysts, to result in a sufficient reaction.[23] However, the rate of H_2O_2 oxidization is decreased with a decrease in flow rates because at that time the amount of the oxidized product in a unit time become less. On the other hand, the reaction between luminol and H_2O_2 is a fast process. When the luminol is sufficient, the intensity of emitted CL is dependent on the rate of H_2O_2 produced from the converter. In conclusion, the slow flow rates are unfavorable for the improvement of sensitivity although they are positive to a sufficient reaction. As shown in Figure 3.5, the CL intensity increased with increasing flow rate in the 0.1-1 ml/min range. When the flow rate is high (>1.5 ml/min), the reaction between luminol and H_2O_2 might be insufficient and as a result the sensitivity of the CL detection system significantly decrease. Finally, a flow rate of 1 ml/min was selected as an optimal condition.

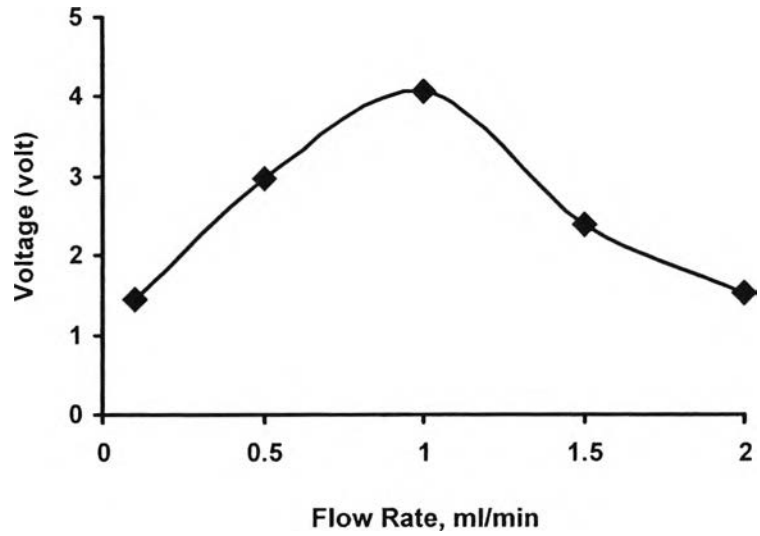


Figure 3.5 Effect of flow rate on CL intensity.

3.4.3.1.2 Effect of NaOH Concentration

The luminol reaction is most efficient at high pH. Figure 3.6 shows an intensity vs NaOH concentration using Cu(II) catalysis. Maximum CL is observed in 0.5 mM of NaOH, while the significant decreased at higher and lower NaOH molar concentrations. The concentration of NaOH is related to the enzymatic activity. Since, the pH adjustment to conditions suitable for observing luminol CL can simultaneously serve as a means of stopping enzyme/catalyzed process.[24] However, NaOH concentration is also an important limitation to applications of luminol CL. High NaOH concentration (in the range of 1-2 mM) may accelerate the rate of reaction between hydrogen peroxide and reducing components in biological samples. These reaction consume hydrogen peroxide before it can react with luminol, thus, reducing observed CL intensity and interfering negatively in analytical procedures.[25]

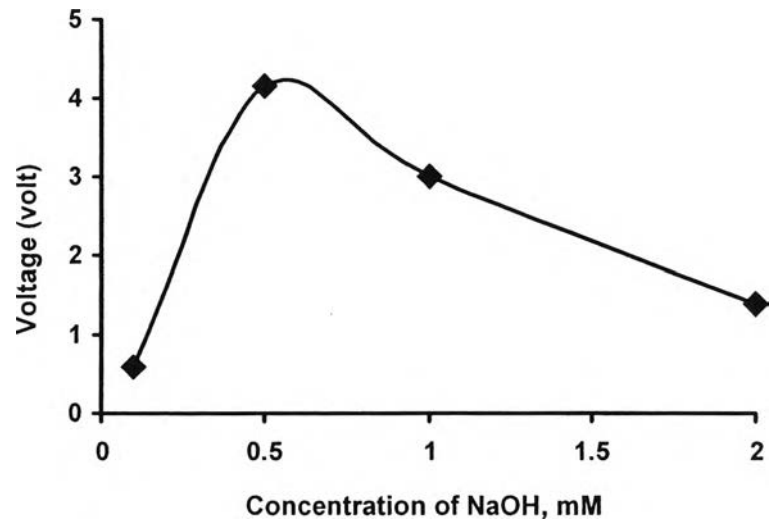


Figure 3.6 Effect of NaOH concentration on CL intensity.

3.4.3.1.3 Effect of H_2O_2 concentration

Figure 3.7 shows the CL intensity as a function of H_2O_2 molar concentrations. The H_2O_2 molar concentrations were varied in the range 5-100 mM. Below 20 mM H_2O_2, light emission is proportional to peroxide concentration. Further increases in peroxide concentration do not appreciably increase light emission. A maximum CL signal was obtained at 20 mM. As a result, the selected hydrogen peroxide molar concentration was 20 mM.

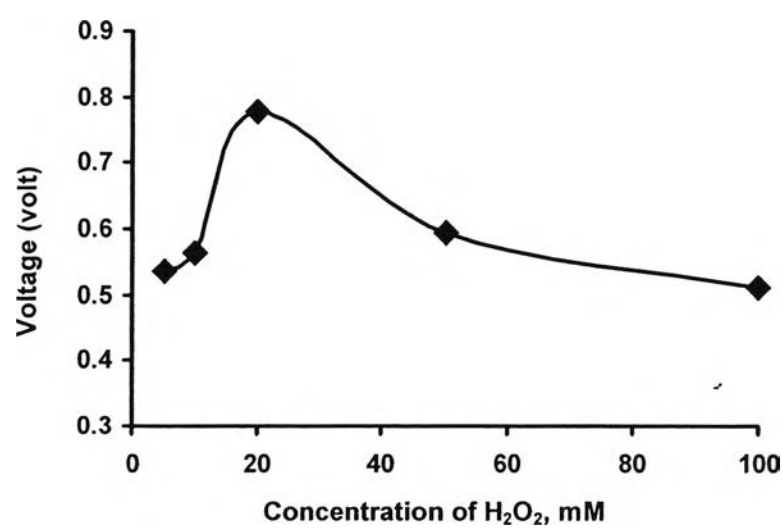


Figure 3.7 Effect of H_2O_2 concentration on CL intensity.

3.4.3.1.4 Effect of 3 concentration

The effect of **3** concentration was investigated as shown in Figure 3.8. The results showed that the optimal concentration of **3** was 2 mM. The substrate concentration is not a critical variable in designing methods based on coupling to peroxide.[23] In applications involving CL induced by complexation of metal ions by organic ligands, it is explicitly and implicitly assumed that only a fraction of the metal ion is complexed by the organic ligand and the CL emission intensity would vary as a fraction of free metal ion.[6] As the concentration of 3 increases, the fraction of the complexed metal ion also increases and the concentration of the free metal ion drops leading to a decrease in the CL intensity.

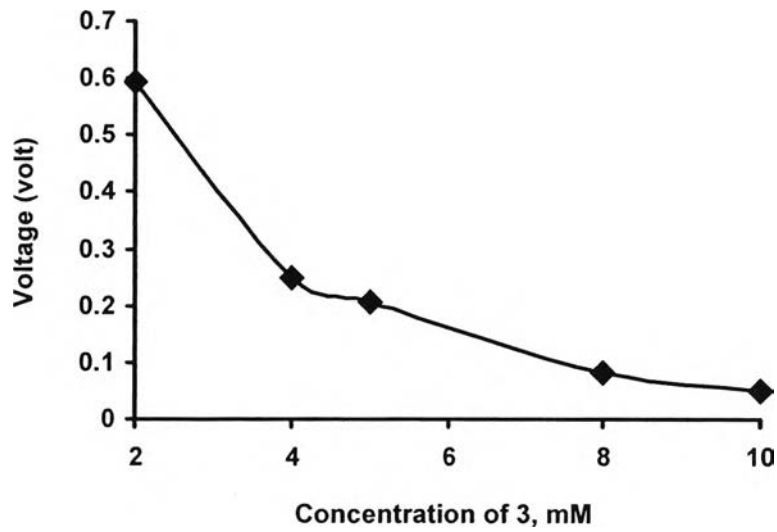


Figure 3.8 Effect of **3** concentration on CL intensity.

3.4.3.2 Effectiveness of difference catalysts

The choice of catalyst is critical for analytical purposes. The catalyst influences the rate of CL reaction, CL efficiency, the order of the reaction and the stoichiometry.[26] Several transition metal ions were reported to catalyze luminol CL. Co(II) and Cu(II) are the most efficient[27] the same trend was shown in Figure 3.9

while there is no significant effect of others metal ion when compare with Co(II) and Cu(II). The mechanism is thought to involve a metal ion-peroxide complex as the active species reacting with luminol.[28] Both Co(II) and Cu(II) have been investigated as catalysts for peroxide using luminol system,[29] however, two problems are encountered. The principal problem with Co(II) is solubility over the pH range from 10 to 12, where luminol CL is most efficient.[27] The solubility can be increased by adding ligands like NH_3 to form soluble complexes, however, this is self-defeating because complexing agents reduce catalytic efficiency by preventing the formation of metal ion–peroxide complex.[30] With Cu(II) as a catalyst, sensitive peroxide analysis is possible, but intensity is proportional to $[H_2O_2]^n$, where n is larger than one. The value of n is quite sensitive to conditions such as pH and luminol concentration. Probably this behavior is in some way due to the 2:1 stoichiometry of the peroxide-luminol reaction.[31] However, as shown in Figure 3.7, the CL intensity of our system independent of $[H_2O_2]$ by using Cu(II) as catalyst. So we can use compound **3** as a novel substrate to solve the problems of using Cu(II) as catalyst in flow injection analysis system.

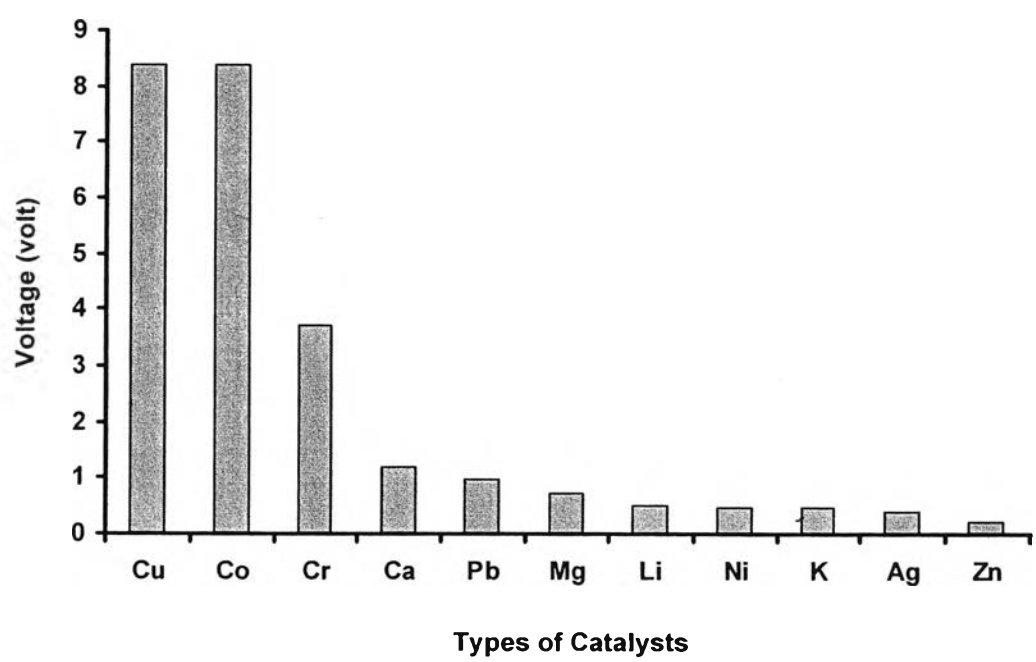


Figure 3.9 CL intensity with various types of catalysts.

3.4.3.3 Response of the CL detection system to Cu(II) Concentration

As mentioned in 3.3.2, compound **3** can form host-guest complex with only Cu(II). Under the selected conditions given above, a linear response in concentration range 0.5 to 3.25 mM was obtained for the determination of Cu(II) as shown in Figure 3.10. The obtained flow injection system is one of the model to determine Cu(II) ion

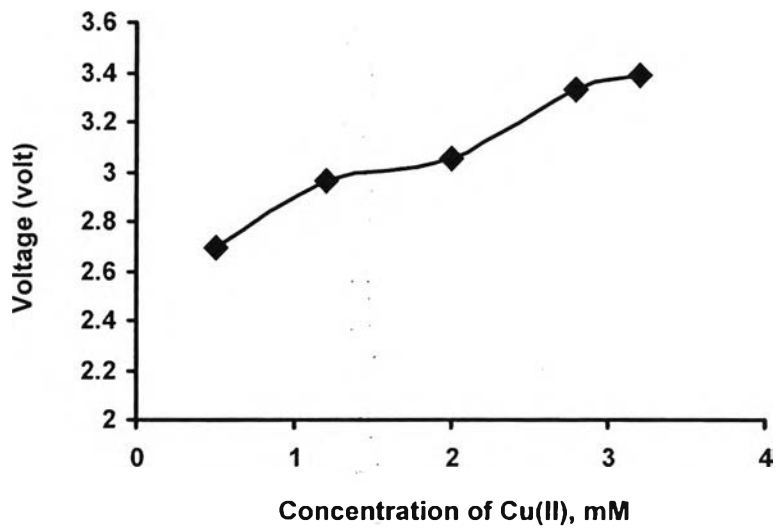


Figure 3.10 CL intensity with Cu(II) concentration.

3.4.3.4 Effect of Chelating Agent on FIA System

Figure 3.11 illustrates the effects of chelators on the CL intensity, studied at a 2:1 molar ratio of chelator to metal. Previously, Arora *et. al.* reported the quenching the fluorescence intensity decreased to approximately 30% of the original value by the addition of EDTA, followed by Fe(II) ions.[32] The same trends were observed when the other three chelators-ADP, NTA, and citrate were added. The factor that may explain the effects of the chelators studied on the fluorescence intensity of metal-catalyzed reaction may be their effect on the redox potential of the metal ions.[32] Transition metals have a range of accessible oxidation states that

enables them to transfer electrons. The redox potential for such a transfer is altered by chelation of the metals.[33] The reduction potential of Fe(III)/Fe(II) (aqueous) is +110 mV at pH 7.0. Whereas those of the chelated Fe(III)EDTA/Fe(II)EDTA, Fe(III)citrate/Fe(II)citrate and Fe(III)ADP/Fe(II)ADP complexes decrease to +100, +90 and +75 mV, respectively at neutral pH.[34] In general, chelators in which oxygen atoms ligate the metal tend to preferentially bind to the oxidized forms of iron or copper, thereby decreasing the redox potential of these metals.[32]

Figure 3.11 shows the CL spactra of **3**-Cu and **3**-Cu-chelating agent (EDTA) to observe the effect of chelating agent on CL signal. The CL spectra of **3**-Cu-EDTA (Figure 3.11b) is much lower than **3**-Cu (Figure 3.11a). This confirms that the complexation of catalyst and chelating agent leads to the decreasing in the CL spectra. The CL compounds that can perform CL properties without adding chelating agent are needed.

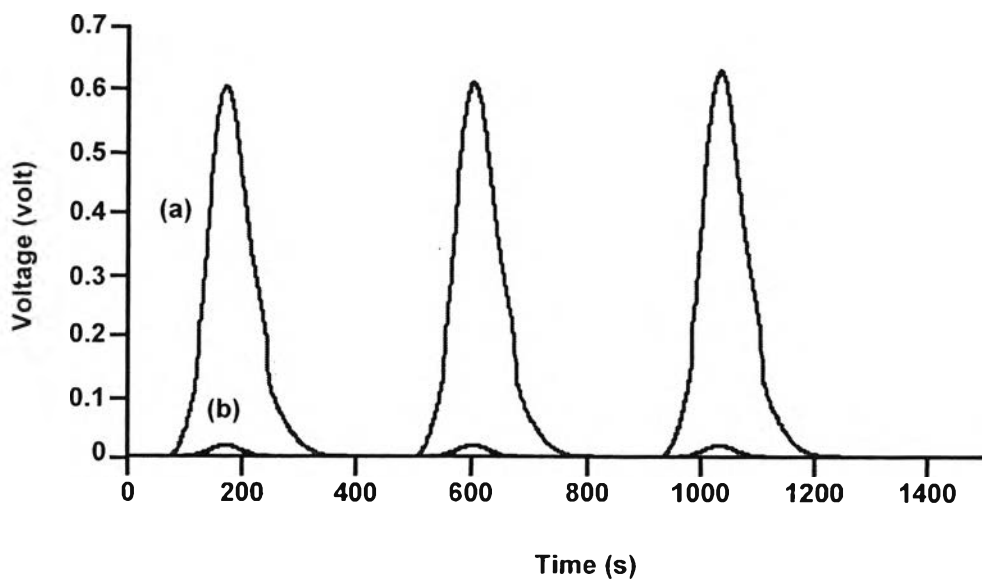


Figure 3.11 Chemiluminescence spectra obtained from triplicate injections of (a) **3**-Cu and (b) **3**-Cu-EDTA.

3.5 Conclusions

A novel chemiluminescent host: 5-((3-(((2-hydroxy-5-methylbenzyl)(methyl)amino)methyl)-2-hydroxy-5-methylphenyl) diazenyl)-2,3-dihydrophthalazine-1,4-dione was successfully prepared by conjugation luminol and *N,N*-bis(5-methyl-2-hydroxybenzyl)methylamine. The obtained compound can form complex with Cu(II) in the 2:1 ratio which can be utilized in the flow injection system resulting in the simple system without adding chelating agent to maintain the CL intensity.

3.6 Acknowledgement

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