Anal. Chem. **2010,** *82,* 2401–2404

1-Ethyl-3-Methylimidazolium Ethylsulfate/Copper Catalyst for the Enhancement of Glucose Chemiluminescent Detection: Effects on Light Emission and Enzyme Activity

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The effect of the ionic liquid 1-ethyl-3-methylimidazolium ethylsulfate ([Emim][$EtSO_4$]) on the copper-catalyzed luminol chemiluminescence (CL) is reported. A drastic light emission enhancement is observed, related to a strong interaction between Cu^{2+} and the imidazolium ring. In these conditions, the CL reaction was able to produce light efficiently at pH as low as 6.5 (amplification factor: $Intensity^{+IL}/Intensity^{-IL} = 2900$). Interesting effects of [Emim][$EtSO_4$] on the enzyme glucose oxidase activity were also evidenced, and advantages were taken from this enhancement to perform sensitive chemiluminescent glucose detection (LOD = 4 μM) at pH 8.0.

The catalyzed chemiluminescent (CL) reaction of luminol has received, for more than 30 years, a great amount of attention[1−6] thanks to its high sensitivity and low background signal,[7,8] properties which make the reaction an attractive analytical chemistry tool.

Luminol CL is initiated by the oxidation of luminol to luminol radical in the presence of strong oxidants at elevated pH. These conditions could be softened through the use of catalysts such as polarized electrodes,[9,10] horseradish peroxidase,[11,12] cobalt, copper, iron cations, DNAzymes,[13] and the corresponding organic complexes of these metals.[14,15] The use of these catalysts usually leads to a decrease of both the optimum reaction pH and the necessary oxidant concentration. Enhancer could also be used in order to obtain increased CL signal.[2,12]

Luminol chemiluminescent reaction catalyzed by metallic cations is known to be optimal at alkaline pH (∼10)[16] which is compatible with few applications focused on separation methods of luminol labeled molecules.[17−19] Nevertheless, when the analytical system is based on biological molecules such as enzymes or binding proteins, this elevated pH happens to be an insoluble constraint and the preferred catalyst turn out to be the peroxidase[20] which can perform CL reaction at lower pH (∼8.5). The consequences are the use of a fragile and expensive molecule, instead of a cost efficient and stable metallic cation, for analytical applications.

In the present study, we introduce the beneficial effect of imidazolium ring-based ionic liquids (Figure 1)[21] on the metal-catalyzed luminol CL reaction, i.e., optimum pH lowering and signal amplification.

The presence of ionic liquids (IL)[22] in oxidation reaction catalyzed by transition metals is well-known to provide significant

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10.1021/ac9026725
Published on Web 02/17/2010

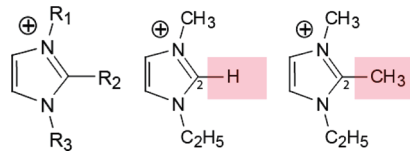

Figure 1. Structures of the imidazolium rings used in this study: general structure (left), [Emim] (center), and [Edim] (right).

advantages in terms of improvement of catalyst stability and activity.[23,24] Moreover, as most IL currently in use are stable to oxidation, they provide ideal solvents for oxidation processes.[25,26]

Besides, copper(II) cations interact and form complexes with imidazole rings and based on structure similarity, ILs made of imidazolium rings (1-ethyl-3-methylimidazolium ethylsulfate and 1-ethyl-2,3-dimethylimidazolium chloride) have been selected to be use as cosolvent in a Cu^{2+} catalyzed luminol CL reaction.

The advantageous new chemiluminescent detection procedure will also be applied to the determination of glucose using glucose oxidase (Gox) as a biological analytical tool. The effect of this IL cosolvent on the enzyme kinetic will be studied and discussed as well.

$$\text{glucose} \xrightarrow{\text{Gox}} \text{glucono-1,4-lactone} + H_2O_2 \qquad (1)$$

$$\text{luminol} + H_2O_2 \xrightarrow{Cu^{2+}} \text{aminophthalate} + h\nu \qquad (2)$$

EXPERIMENTAL SECTION

Materials. 1-Ethyl-3-methylimidazolium ethylsulfate ([Emim][$EtSO_4$]) (99%) and 1-ethyl-2,3-dimethylimidazolium chloride ([Edim][Cl]) were purchased from Alfa Aesar (Germany) and Fluka (Switzerland), respectively. Glucose oxidase (Gox, grade I, EC 1.1.3.4, from *Aspergillus niger*) was obtained from Roche (Germany). Luminol (3-aminophthalhydrazide) and sodium phosphate monobasic monohydrate (99.6%) were supplied by Sigma (France). A stock solution of luminol (5.5 mM) was prepared by dissolving luminol in a 10^{-2} M potassium hydroxide solution (Prolabo, France). Hydrogen peroxide (30%), disodium hydrogenophosphate (99%), and copper(II) dichloride (99%) were purchased from Prolabo (France). All dilutions were made in pure water (Milli-Q Plus system, Millipore, 18.2 MΩ cm^{-1}). The different phosphate buffers (pH 6.5, 7.5, and 8.0) were prepared by mixing the appropriate amount of Na_2HPO_4 (0.1 M) and NaH_2PO_4 (0.1 M).

Caution: Acetate copper is harmful for the environment and causes eye damage. Ionic liquids are irritants for skin, eyes, and airways. The use of these products must be performed with appropriate materials according to safety considerations.

Methods. CL assays were performed in 96 well microtiter plates (Black MaxiSorp, Nunc, Denmark) and monitored using a Luminoskan (Ascent, Labsystems, France).

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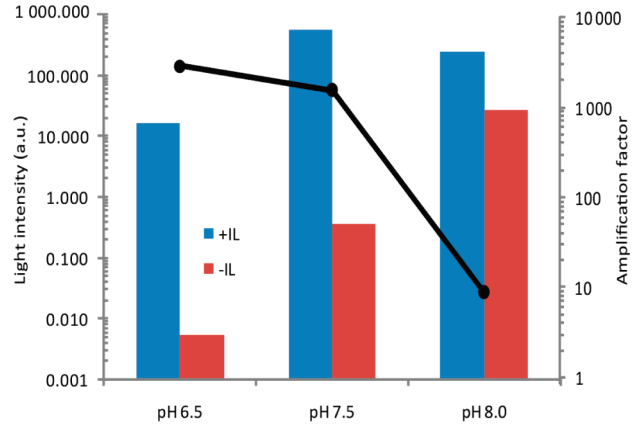

Figure 2. Maximum light intensity at pH 6.5, 7.5, and 8.0 in the presence or in the absence of 1.47 M of [Emim][$EtSO_4$] (optimized amount from Figure 3). CL reaction conditions: luminol, 300 μM; H_2O_2, 1 mM; and Cu^{2+}, 1.67 mM. Calculated amplification factor: filled circles.

Assays were performed in a 0.1 M phosphate buffer with a 300 μL final volume. Briefly, wells were filled with 150 μL of an aqueous solution of IL, copper, and luminol at particular concentrations, and 150 μL of a solution of complementary reagents was injected to initiate the light emission. A maximum CL signal was obtained after 40 s of measurement. The CL signal kinetics is identical in the presence and in the absence of IL. The signal increases rapidly for 1 s to attain its maximum value and then decreases gradually and disappears after 50 s.

The activity of the glucose oxidase was measured using a hydrogen peroxide amperometric sensor from INCELTECH-SGI (France). The H_2O_2 probe was composed of a platinum working electrode polarized at +650 mV vs a platinum pseudoreference electrode. The probe was connected to a PRGE type polarograph (Tacussel, France). The activity measurements were performed in the presence of various glucose concentrations, and the kinetic parameters were extracted from the Eadie-Hofstee linearization of the $V = f([S])$ curves.

RESULTS AND DISCUSSION

Effect of the [Emim][$EtSO_4$] on the Cu^{2+} Catalyzed Luminol/H_2O_2 CL Reaction. Cu^{2+} catalyzed luminol/H_2O_2 CL reaction is well-known to be optimum at elevated pH (at least 10).[16] Nevertheless, working with glucose oxidase for glucose detection inevitably requires the lowering of the pH to more neutral pH values.

The effect of the presence of [Emim][$EtSO_4$] was, thus, studied at pH ranging from 6.5 to 8. Figure 2 presents the chemiluminescent signals obtained for three different pH values in the presence and in the absence of IL. As a matter of fact, an enhancement of the CL signal occurred in the presence of [Emim][$EtSO_4$] with a maximum amplification factor of 2900 at pH 6.5. As a control experiment, the same measurements were performed in the absence of catalyst (Cu^{2+}). In that latter case, no measurable CL signal was observed. Interestingly, the presence of [Emim][$EtSO_4$] was also tested on the chemiluminescent reaction catalyzed by other metallic cations (Fe^{2+} and Ni^{2+}). In these cases, no enhancement of the CL signal occurred. These results are explained by a difference of redox potential between the three metallic cations (Cu^{2+}: +0.159 V;

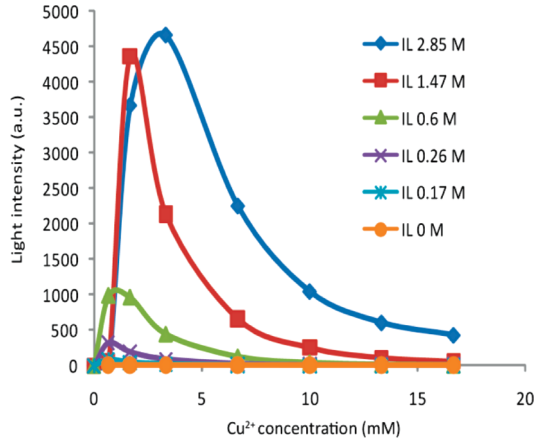

Figure 3. Light intensity as a function of different copper and IL concentrations in phosphate buffer (0.1 M, pH 8.0). Luminol, 300 μM; H_2O_2, 1 mM.

Ni^{2+}: −0.257 V; and Fe^{2+}: −0.44 V vs NHE), Cu^{2+} having the most oxidizing one.

When looking closely to the CL signals obtained, the pH seems to have a drastic effect on the CL enhancement. Indeed, the amplification factor ($Intensity^{+IL}/Intensity^{-IL}$) decreases with the pH from 2900 (pH 6.5) to 1565 (pH 7.5) and then 9 (pH 8.0). Besides this high amplification factor at pH 6.5, the maximum light intensity value was obtained at pH 7.5 in the presence of IL.

This pH dependency of the CL enhancement is assumed to be related to an interaction taking place between Cu^{2+} and [Emim][$EtSO_4$], as already suggested in previous studies,[27] which might be reliant to the presence of the labile proton in the C_2 position of the imidazolium ring.[28−30]

When this labile proton is exchanged by a methyl moiety ([Edim], Figure 1), the entire enhancement properties of the IL are eradicated, whatever the pH used. These results point out the importance of this hydrogen atom position in the assumed interaction between copper cation and imidazolium ring.

Since the CL enhancement observed appears to be dependent on the presence of a putative preformed [Emim][$EtSO_4$]/Cu^{2+} complex, a study of the stoichiometry of this complex formation has been performed. For that purpose, various concentrations of copper and IL were mixed and used to catalyze the luminol/H_2O_2 CL reaction at pH 8.0.

The results are presented in Figure 3 and demonstrate a clear interdependency of the two protagonists' concentration. A most favorable stoichiometry of 850/1 ([Emim][$EtSO_4$]/Cu^{2+}) between the two reagents can here be calculated. Similar results were obtained with pH 6.5 and 7.5 (data not shown).

In the light of the, herein, obtained results, a hypothesis based on a possible interaction can be proposed. On the basis of the heme and metalloporphyrin structures[31] (efficient CL catalysts), an organized [Emim][$EtSO_4$]/Cu^{2+} complex can be suggested in the presence of phosphate,[32] leading to an enhanced Cu^{2+} CL catalysis. To date, the exact structure of this complex remains unknown, even after infrared spectroscopy and NMR studies.

Table 1. H_2O_2 Detection Range, Detection Limit, and Background Values in the Presence or in the Absence of IL at Different Working pHs[a]

condition	pH	detection range (M)	detection limit (M)	background (au)
+IL	8.0	1.10^{-5}−1.10^{-2}	1.10^{-5}	0.021
+IL	7.5	1.10^{-5}−1.10^{-1}	5.10^{-6}	0.0011
+IL	6.5	1.10^{-4}−1.10^{-1}	1.10^{-4}	0.002
−IL	8.0	1.10^{-4}−1.10^{-1}	1.10^{-4}	0.0015
−IL	7.5	1.10^{-3}−1.10^{-1}	1.10^{-3}	0.001
−IL	6.5	1.10^{-2}−1.10^{-1}	1.10^{-2}	0.0035

[a] [Emim][$EtSO_4$]/Cu^{2+}, 850/1; luminol, 300 μM.

Hydrogen Peroxide Detection. The [Emim][$EtSO_4$]/Cu^{2+} (850/1) catalyst was applied to the detection of H_2O_2. Concentrations ranging from 20 mM to 2 nM were tested in the presence of luminol (300 μM). The obtained CL signal intensities were compared to CL signal intensities achieved in the presence or absence of IL. The analytical characteristics (detection range, detection limit, and background signal) of the hydrogen peroxide detection at different pH values are presented in Table 1. Background signal is reflecting the catalyzed CL reaction in the absence of the specific oxidant (H_2O_2), a signal which might interfere with the specific detection.

As expected and according to the amplification factors presented in Figure 2, the [Emim][$EtSO_4$]/copper catalyst enables a more sensitive H_2O_2 detection when compared to the copper catalyst alone. Moreover, the [Emim][$EtSO_4$]/copper catalyst used at pH 7.5 was found to generate the most efficient detection with a detection limit of 5 μM and a detection ranging over 4 orders of magnitude.

Interestingly, the background signals, do not evidence significant changes with the presence of IL except at pH 8.0. This point has a direct effect on the pH 8.0 detection limit which was only 10 μM. Such performances of the hydrogen peroxide detection using luminol chemiluminescent reaction are comparable with previously published results using immobilized biocatalyst[33] or electrochemically catalyzed reaction.[9]

Glucose Detection and Enzyme Catalysis in [Emim][$EtSO_4$]. The advantageous H_2O_2 chemiluminescent detection using [Emim][$EtSO_4$]/copper as catalyst has been applied to the detection of glucose using glucose oxidase (Gox). Figure 4 presents the calibration curves obtained at different pH values in the presence or in the absence of [Emim][$EtSO_4$]. Surprisingly, the pH 8.0 condition in the presence of IL was giving the best glucose detection with a limit of detection of 4 μM and a detection ranging over at least 2 orders of magnitude. This difference between the hydrogen peroxide detection results and the glucose detection optimum was first attributed to a possible variation of the glucose oxidase kinetic parameters.

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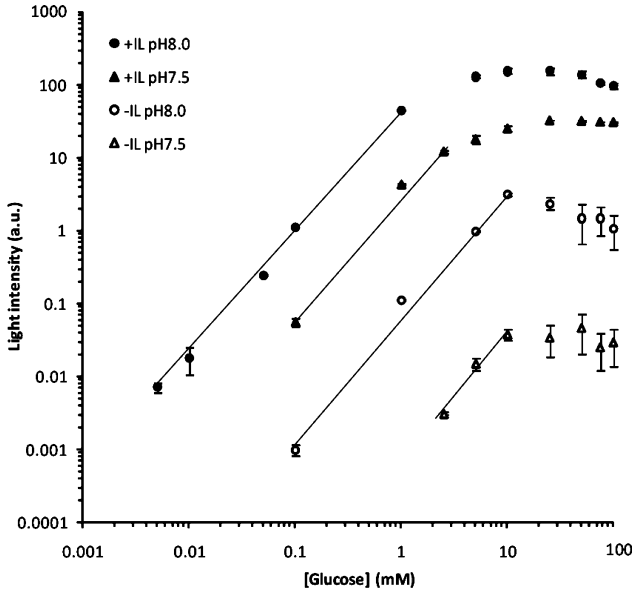

Figure 4. Glucose detection using 1 IU/well of Gox, in the presence or in the absence of IL in phosphate buffer (0.1 M, pH 8.0 or pH 7.5). [Emim][$EtSO_4$]/Cu^{2+}, 850/1; luminol, 300 μM.

Table 2. Influence of IL Presence in the Reaction Buffer on the Enzyme Catalytic Parameters (M_{Gox} = 160 000 g/mol; Gox, 1.14 μg/mL (1 IU/assay); Phosphate Buffer, 0.1 M)

condition	V_{max} (μmol/min/mg)[a]	K_m (mM)[a]	V_{max}/K_m (M^{-1} s^{-1})
−IL, pH 7.5	172.08	26.16	17540
+IL, pH 7.5	25.56	5.36	19420
−IL, pH 8.0	441.15	90.45	13006
+IL, pH 8.0	9.61	2.00	12817

[a] Calculated from the Eadie-Hofstee linearization of the $V = f([S])$ curves.

An enzyme kinetic study has then been performed through the electrochemical measurement of the H_2O_2 produced during the course of the catalyzed reaction (Reaction 1), using a Pt/Pt electrochemical sensor (+650 mV). The results obtained are presented in the Table 2. As can be seen, the kinetic parameters of the enzyme are quite different in the presence and in the absence of IL. Indeed, a drastic lowering of the Michaelis constant (K_M) together with a decrease of the maximum velocity (V_{max}) were observed at both pH values. This concomitant variation led to relatively unchanged catalytic efficiencies (V_{max}/K_m). Thus, changing pH or buffer composition (presence of IL) did not impact significantly the efficiency of the biocatalyzed reaction. The expected changes of enzyme activity, which should have explained the very good glucose detection at pH 8.0, were herein not observed.

A credible explanation to these good results at pH 8.0 is the possible formation of IL microdomains in the aqueous solution, leading to an increase of the viscosity of the bulk solution and consequently to a diffusion limitation of products from the aqueous microenvironment of the biocatalyst leading to a local product overconcentration. In this microenvironment, the glucose oxidase reaction product glucono-1,4-lactone is hydrolyzed to gluconic acid, and this weak acid, when accumulated, can impact on the pH microenvironment.[9] It is then worth thinking that if such pH variations occurred, the observed CL intensities at pH 8.0 were actually CL signals measured at lower pH.

CONCLUSION

This paper describes for the first time the effect of an IL on the Cu^{2+} catalyzed luminol chemiluminescence in solution. This reaction is classically performed in basic conditions (pH 11). IL addition to the reaction buffer allows the lowering of the reaction pH to 7.5 while preserving the reaction efficiency toward hydrogen peroxide detection. In these conditions, the batch detection of H_2O_2 was as efficient as the best previously published results using immobilized biocatalyst[33] or electrochemically catalyzed reaction.[9] A strong interaction between Cu^{2+} and IL was believed to generate a new CL catalyst with enhanced performances.

When the IL-enhanced CL reaction in a coupled reaction with glucose oxidase was used, interesting effects were also observed. First the enzyme was able to catalyze its specific reaction in the presence of 17% (v/v) of [Emim][$EtSO_4$]. The kinetic parameters of the enzyme were modified, but the catalytic efficiency remained stable.

Then, when the coupled reaction for the detection of glucose was used, unexpected good performances at pH 8.0 were obtained, evidencing a complex effect of the IL (microdomains) onto the coupled reaction within the reaction buffer. This observation was correlated to a possible local pH modification due to the accumulation of acidic reaction product.

No doubt that such effect on enzyme catalysis at high IL concentration might in the future be studied more deeply, by our group and others, and that the presently observed phenomenon can be soon fully understood. In, these optimized conditions, the glucose detection performances (LOD of 4 μM) of the [Emim]-[$EtSO_4$]/Cu^{2+} system were found to be as good as the ones obtained using immobilized peroxidase[33] or electro-generated chemiluminescence.[9] Nevertheless, the present system was found much simpler to produce and use. Its extension to other H_2O_2 based analyte detection (lactate, cholesterol, glutamate, choline) is now under study.

Received for review November 23, 2009. Accepted February 4, 2010.

AC9026725