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Stainless steel electrode for sensitive luminol electrochemiluminescence detection of H$_2$O$_2$, glucose, and glucose oxidase activity

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ABSTRACT: The electrogenerated chemiluminescence (ECL) application of stainless steel, a robust and cost-effective material, has been developed for the first time. The type 304 stainless steel electrode shows appealing ECL performance for luminol-H$_2$O$_2$ system. It enables the detection of H$_2$O$_2$ with a linear range from 1 to 1000 nM and a limit of detection of 0.456 nM (S/N = 3). The ECL method based on type 304 stainless steel electrode is more sensitive, more cost-effective, and much simpler than other ECL methods reported before. Because the stainless steel electrode has excellent performance for H$_2$O$_2$ detection and H$_2$O$_2$ participates in many important enzymatic reactions, the applications of stainless steel electrode-based ECL for the detection of enzyme activities and enzyme substrates were further investigated using glucose oxidase (GODx) and glucose as representative enzyme and substrate. The concentrations of glucose and the activity of GODx were directly proportional to the ECL intensities over a range of 0.1 – 1000 µM and 0.001 – 0.7 U/mL with a limit of detection of 0.076 µM and 0.00087 U/mL (S/N = 3), respectively. This method was successfully used for determining glucose in honey. Because of its remarkable performance and user-friendly features, stainless steel electrode holds great promise in various electroanalytical applications, such as biosensing, disposable sensors, and wearable sensors.

Electrogenerated chemiluminescence (ECL) is one of the most powerful analytical techniques and has widespread applications in various research areas. ECL is a well-known method depending on the generation of an optical signal by an electrochemical reaction. An increased interest is observed for ECL investigations because of outstanding advantages, such as high sensitivity, selectivity, the absence of background signal, easiness to control the reaction by applying potential at the electrode, wide linear range and so on. Due to this fact, ECL method has been used for wide analytical applications.

Luminol is one of the well known luminophores. Because of high sensitivity for H$_2$O$_2$, most of the applications of luminol-H$_2$O$_2$ ECL are used to detect H$_2$O$_2$ generating label enzymes, H$_2$O$_2$ enzymatic precursors, and luminol-labelled molecules. Luminol can generate strong ECL signal with H$_2$O$_2$, which takes part in many enzymatic reactions. Due to this, luminol ECL plays significant role in the determination of H$_2$O$_2$, enzymes, and substrates that can be converted to H$_2$O$_2$ enzymatically and H$_2$O$_2$-related biosensors. For example, luminol ECL has been used for the determination of glucose and glucose oxidase (GODx) since glucose can react with dissolved oxygen to produce H$_2$O$_2$, in presence of GODx. ECL of luminol has been studied at different electrodes, such as glassy carbon, platinum, gold, copper, paraffin-impregnated graphite electrodes. These previous works clearly indicated that the electrode material plays highly important role in the ECL of luminol.

Stainless steels are iron alloys which contain more than 10% chromium by mass and other alloying elements to enhance the properties of steel and corrosion resistance. Besides its corrosion resistance, stainless steel has interesting features, such as environmentally friendly, low-cost, good electrical conductivity, high mechanical strength, and commercial availability. To the best of our knowledge, stainless steel has never been used as working electrode for ECL study.

In this study, stainless steel has been explored as working electrode for ECL study for the first time. The ECL behaviors of luminol at stainless steel electrode were studied. Its use for the detection of luminol, H$_2$O$_2$, glucose and GODx were investigated. The use of stainless
Steel electrode significantly enhances the sensitivity for the detection of \( \text{H}_2\text{O}_2 \), glucose and GODx.

![Graph](image)

**Figure 1.** ECL intensity curves (A) and Linear sweep voltammograms (B) of blank (a), 0.5 mM luminol (b) and 0.5 mM luminol and 0.5 mM \( \text{H}_2\text{O}_2 \) (c) measured at the type 304 stainless steel electrode in 0.1 M carbonate buffer solution pH 11.0; Scan rate: 100 mV s\(^{-1}\); PMT voltage = 600.

**EXPERIMENTAL SECTION**

**Chemicals**

We bought types 201, 304, and 316 stainless steel from Suzhou Qiangda Fastener Co., Ltd. Luminol and hydrogen peroxide were obtained from Beijing Chemical Reagent Company. Glucose oxidase was purchased from Sigma. β-D-Glucose (>99.8%) was purchased from Amresco. Ethiopian highland honey was used for real sample analysis. 0.1 M phosphate buffer solution (pH 8 and 9) and 0.1 M carbonate buffer solution (pH 10, 11 and 12) were prepared by mixing a balanced amount of 0.2 M NaH\(_2\)PO\(_4\) and 0.2 M NaH\(_2\)PO\(_4\) and 0.2 M Na\(_2\)CO\(_3\) and NaHCO\(_3\), respectively. Stock solution of 10 mM luminol was prepared by dissolving luminol in 0.1 M NaOH. Luminol working solutions were prepared by diluting the stock solution with 0.1 M carbonate buffer solution (pH = 11). Glucose (10 mM) and GOD\(_x\) (10 mg/mL) were prepared in phosphate buffer solution (PBS) (pH = 7.4) and double distilled water, respectively and stored at 4°C when not in use. Working solutions of glucose were prepared by diluting the stock solutions with PBS. Working solution in the cell was changed with fresh solution at each time. All experiments were carried out at room temperature.

**Apparatus**

Electrochemical measurements were carried out in a conventional three-electrode system with a CHI 660C electrochemistry workstation and ECL measurements were performed with a BPCL-1-KIC luminescence analyzer, and unless noted otherwise, the photomultiplier tube (PMT) was biased at 900 V. Stainless steel electrode (3 mm in diameter), Ag/AgCl (saturated KCl) electrode and a gold wire were used as working, reference and auxiliary electrode, respectively. The stainless steel electrode was fabricated by tightly pack stainless steel wire with 3 mm in diameter into the electrode cavity of Teflon tube. The stainless steel electrode was polished with abrasive papers and slurry of 0.03 µm alumina then sonicated and rinsed with double distilled water. The ECL measurement for glucose biosensing

A desired amount of glucose (pH 7.4) and GOD\(_x\) were mixed in a test tube and allowed to react for 5 minutes. Then 200 µL of 5 µM luminol (pH 11) was added and the volume was adjusted to 1 mL by adding carbonate buffer solution (pH 11). A desired volume of the working solution was added into the ECL cell. ECL signals were generated, when the potential was applied to the working electrode. The ECL intensity vs time (\(I_{\text{ECL}}/t\)) and current vs potential (i/E) curves were recorded.

**RESULTS AND DISCUSSION**

**ECL and electrochemical studies of luminol–\( \text{H}_2\text{O}_2 \) system at the stainless steel electrode**

Figure 1A shows the ECL curve of luminol with and without \( \text{H}_2\text{O}_2 \). The ECL emission was observed approximately at 0.70 V vs Ag/AgCl for luminol-\( \text{H}_2\text{O}_2 \) and 0.8 V vs Ag/AgCl for luminol system. The ECL intensity of the luminol-\( \text{H}_2\text{O}_2 \) system is approximately 26 times stronger than that of only luminol at type 304 stainless steel electrode. This is because \( \text{H}_2\text{O}_2 \) could generate reactive oxygen species (ROS) upon the anodic potential scanning at the electrode, which can accelerate the ECL reaction of luminol with enhanced ECL intensity. As shown in Figure 1B the anodic peak current of luminol-\( \text{H}_2\text{O}_2 \) system is greater than the anodic peak current of only luminol. Generally, anodic luminol ECL is weak in the absence of \( \text{H}_2\text{O}_2 \) due to the difficulty to produce ROS, and requires the addition of \( \text{H}_2\text{O}_2 \). To give insight into the ECL on the stainless steel electrode, we have also tested ECL at other type stainless steel electrodes. As shown in Figure S1, the ECL intensities at types 201, 304 and 316 stainless steel electrodes are similar. All the stainless
stainless steels contain much chromium. It is well-documented that the surface of stainless steel is covered with a thin layer of chromium (III) oxide. It suggests that the thin layer of chromium (III) oxide of stainless steel play critical roles for the enhanced ECL properties on stainless steel electrode. Since type 304 stainless steel is cost-effective and widely used, we employed type 304 stainless steel electrode for following experiments.

The effect of scan rate on the oxidation peak current and ECL intensity of luminol-H$_2$O$_2$ system was also studied. As shown in Figure 2A and B, the oxidation currents and ECL intensities are proportional to the square root of the scan rate over a range of 10 to 400 mVs$^{-1}$, demonstrating that the electron transfer reaction is controlled by the diffusion of luminol and H$_2$O$_2$.

Since the luminol is the luminophore, the emission mechanism was proposed as follows. Luminol is first oxidized at the electrode to luminol anion (Equation 1 of Scheme S1) upon the anodic potential scanning at type 304 stainless steel electrode. Then, luminol anion reacted with ROS generated by H$_2$O$_2$ (Equation 3 of Scheme S1) to produce the excited state luminol anion which emits light with $\lambda_{max}$~425 nm (Equation 4 of Scheme S1).

**Effect of pH for luminol – H$_2$O$_2$ ECL**

The ECL intensities of the 10 µM luminol and 10 µM H$_2$O$_2$ was studied in phosphate buffer solution (pH 8.0 and 9.0), and carbonate buffer solution (pH 10.0, 11.0 and 12.0) at type 304 stainless steel electrode. As shown in Figure S3A, the ECL intensities increase with increasing pH value from 8.0 to 11.0 and then decrease as the pH increases further. ECL of luminol-H$_2$O$_2$ system reactions are involved in the electrochemical oxidation of luminol and electrogeneration of ROS from H$_2$O$_2$. Therefore, the increase of ECL intensities from pH 8.0 to 11.0 is attributed to the faster generation of ROS and the deprotonation of luminol.

**Detection of luminol and H$_2$O$_2$**

The ECL intensity has a linear relationship over luminol in range of 10 to 1000 nM when the concentration of H$_2$O$_2$ is 10 µM. Also, under optimal conditions, the ECL intensity has a linear relationship over H$_2$O$_2$ in the concentration range between 1 to 1000 nM when the concentration of luminol is 10 µM and the detection limit of luminol and H$_2$O$_2$ is 2.41 and 0.456 nM (S/N = 3), respectively (Supporting Information Figure S4A and B). The linear range and the detection limit of H$_2$O$_2$ determination at type 304 stainless steel electrode were compared with other reported ECL methods in Table S1. It can be seen from the table that our method has wider linear range and higher sensitivity. The relative standard deviations (RSD) for 5 repetitive measurements of 10 nM luminol and 10 nM H$_2$O$_2$ were of 5.76 and 3.23%, respectively.

**ECL behaviors of luminol–glucose–GODx system at the stainless steel electrode**

Because glucose can be oxidized by GODx to generate H$_2$O$_2$, and this enzymatic reaction has been extensively used for the detection of glucose and GODx, the ECL intensities of luminol at type 304 stainless steel electrode with and without glucose-GODx are compared and the results are shown in Figure 3. The ECL intensity of the luminol-glucose-GODx system is approximately 27 times stronger than that of only luminol at type 304 stainless steel electrode. As shown in Scheme 1. First, H$_2$O$_2$ is generated from the oxidation of glucose in the presence of GODx. Second, under positive potential scan luminol and enzymatically generated H$_2$O$_2$ undergoes oxidation to produce luminol radical anion and ROS, respectively. Finally, the produced luminol radical anion and ROS...
react to generate the excited-state 3-aminophthalate anion which emit light (λ<sub>max</sub> = 425 nm) on relaxation to the ground state.

![Figure 3](image)

**Figure 3.** ECL intensity curves of 1 µM luminol (a) and 1 µM luminol – 1 mM glucose – 1 mg/mL GODx (b) measured at the type 304 stainless steel electrode.

**Optimizing the performance of the ECL biosensor**

In order to optimize the performance of the proposed ECL biosensor towards glucose and GODx detection, the effect of pH, the effect of reaction time, and the effect of concentration of GODx were studied.

**Scheme 1.** Possible mechanism of the luminol-glucose-GODx system on the stainless steel electrode

**Effect of pH for glucose–GODx reaction**

The effect of solution pH of glucose-GODx was studied from 7.0 to 8.0 on the biosensor performance. The ECL intensities increase with increasing pH value from 7.0 to 7.4 and then decrease as the pH increases further to 8.0 (Figure S3B). It is well-known that the pH of a solution has significant effect on the activity of GODx. Due to less activity of GODx at higher pH values, the amount of H<sub>2</sub>O<sub>2</sub> produced from the catalytic reaction of glucose and dissolved oxygen will be less and thus the ECL intensity decreased. Therefore, a PBS at pH 7.4 was selected as the supporting electrolyte for the reaction of glucose and GODx in the further studies.

**Effect of reaction time for glucose – GODx ECL**

The effect of reaction time of glucose and GODx on the ECL intensity is also studied (Figure S3C). With increasing the reaction time, ECL intensity increased and did not show significant increase after 5 minutes of reaction time. To obtain good sensitivity and save measurement time, a reaction time of 5 minutes was selected for the following experiments.

**Effect of GODx activity on Glucose–GODx ECL and Determination of GODx activity**

Figure 4 and Figure S3D show the effect of activity of GODx on the ECL signals. There is a good linear relationship between the ECL intensities and GODx activity from 0.001 U/mL to 0.7 U/mL with a regression equation \(I_{ECL} = 7407.5 \times + 367.5\) and a correlation coefficient of 0.991 (n= 8), where \(x\) is the GODx activity in U/mL. The detection limit is 0.00087 U/mL. The method is more sensitive than the previous studies.\(^{36-38}\) The ECL intensities increase further with increasing GODx activity and level off at an GODx activity of 5 U/mL. Thus, 5 U/mL of GODx was chosen for the determination of glucose.

![Figure 4](image)

**Figure 4.** ECL intensity curves of type 304 stainless steel electrode at different activities of GODx and the inset is the calibration curve for GODx activity.

**Glucose detection based on ECL biosensor**

The ECL biosensor was evaluated under the optimized experimental parameters by determining standard glucose solutions. As shown in Figure 5, the ECL intensities increase with increasing glucose concentrations. The linear calibration range is from 0.1 µM to 1000 µM. The regression equation is \(I_{ECL} = 8.724 \times + 660.406\) with a correlation coefficient of 0.989 (n= 9), where \(C\) is the glucose concentration in µM. The detection limit (S/N=3) for glucose is 0.076 µM. The relative standard deviation of the biosensor response to 0.3 mM glucose was 4.23% for eight successive measurements (Figure S5). As shown in Table 1, this method exhibits higher sensitivity than most previous reported methods. Moreover, the electrode used is much
simpler, cheaper and more robust than that used in other ECL methods.

Figure 5. ECL intensity curves of type 304 stainless steel electrode at different concentrations of glucose and the inset is the calibration curve of glucose.

Interference study of the ECL biosensor

Some species which co-exist and possibly interfere with glucose detection were tested. Increasing amounts of interfering species were added into the solution containing 0.3 mM of glucose. An interfering specie was considered not to interfere if it caused a relative error < 5% during the measurement of 0.3 mM glucose sample. Figure S6 shows that 10 µM ascorbic acid, 5 µM dopamine, 10 µM uric acid, 5 mM fructose, 5 mM galactose and 5 mM phenylalanine have negligible interference with the determination of 0.3 mM glucose.

Table 1: Comparison of the analytical performance of proposed glucose detection method with some luminol based ECL glucose biosensors

<table>
<thead>
<tr>
<th>ECL System</th>
<th>Linear range (µM)</th>
<th>LOD (µM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLANC</td>
<td>0.1 – 50</td>
<td>0.08</td>
<td>39</td>
</tr>
<tr>
<td>SG/GCE</td>
<td>50 - 10000</td>
<td>26</td>
<td>40</td>
</tr>
<tr>
<td>MWNT/ NF/GCE</td>
<td>5 – 800</td>
<td>2</td>
<td>41</td>
</tr>
<tr>
<td>PtNFs/GO/GODx</td>
<td>5 – 80</td>
<td>2.8</td>
<td>42</td>
</tr>
<tr>
<td>AuNFs/SG/Au electrode</td>
<td>1 – 5000</td>
<td>0.2</td>
<td>43</td>
</tr>
<tr>
<td>Pd NP/MWCNT/GCE</td>
<td>0.1 – 1000</td>
<td>0.054</td>
<td>44</td>
</tr>
<tr>
<td>GR/NF/GOD/GCE</td>
<td>2 – 100</td>
<td>1</td>
<td>45</td>
</tr>
<tr>
<td>MWCNT/AuNFs/GOX/CS/GCE</td>
<td>1 – 1000</td>
<td>0.5</td>
<td>46</td>
</tr>
<tr>
<td>CNP</td>
<td>1 – 2000</td>
<td>0.5</td>
<td>47</td>
</tr>
<tr>
<td>CCCE</td>
<td>10 - 10000</td>
<td>8.16</td>
<td>48</td>
</tr>
<tr>
<td>Type 304 stainless steel electrode</td>
<td>0.1 – 1000</td>
<td>0.076</td>
<td>This work</td>
</tr>
</tbody>
</table>

Table 2: Determination results of glucose in real sample (n=5)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Added (mM)</th>
<th>Found (mM)</th>
<th>%RSD</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honey</td>
<td>-</td>
<td>0.309</td>
<td>6.07</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>0.434</td>
<td>3.92</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>0.524</td>
<td>2.76</td>
<td>102.9</td>
</tr>
<tr>
<td></td>
<td>0.35</td>
<td>0.634</td>
<td>4.89</td>
<td>96.2</td>
</tr>
</tbody>
</table>

Real sample analysis

The proposed method has been applied to evaluate glucose content in honey samples without sample pretreatment except a dilution step. ECL of 1 mg/mL honey solution in 0.1 M PBS pH 7.4 was used to determine glucose under optimized condition and the results are shown in Table 2. The percent recovery values range from 96% to 103%, indicating that the present methods is feasible for real sample analysis.

Conclusion

The ECL application of stainless steel working electrode has been demonstrated for the first time using luminol-H$_2$O$_2$ system as representative system. The stainless steel working electrode shows superior sensitivity for H$_2$O$_2$ detection in comparison with ECL methods reported. By coupling with enzymatic reaction, it allows sensitive detection of glucose and GODx activity. Since stainless steel has excellent performance, as well as is cheap, robust and user-friendly, it is a highly promising electrode for ECL applications, such as ECL biosensing, disposable sensors, and wearable sensors.

ASSOCIATED CONTENT

Supporting Information

Additional information about ECL intensity curves luminol and luminol-H$_2$O$_2$ measured at the 201, 304 and 316 stainless steel electrodes, Reaction mechanism for the luminol-H$_2$O$_2$ ECL system, the ECL spectrum of luminol-H$_2$O$_2$ system at stainless steel electrode, effect of experimental parameters, linear calibration curve for luminol and H$_2$O$_2$, comparison of linear range and detection limit with different ECL system toward H$_2$O$_2$ and interference study. (pdf)

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