Original Papers

**Water soluble Hemin-mPEG-enhanced luminol chemiluminescence for sensitive detection of hydrogen peroxide and glucose**

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Abstract

In the present study, we synthesized a water-soluble substance (Hemin-mPEG) at room temperature by using hemin and poly(ethylene glycol) methyl ether (mPEG). It was found that the Hemin-mPEG maintained the excellent catalytic activity inheriting from hemin and was first used to catalyze luminol–H₂O₂ chemiluminescence (CL) system to generate intense and slow CL signal. The results of the mechanism research showed that the presence of Hemin-mPEG could promote the production of oxygen-relative radicals from H₂O₂ and dissolved oxygen in solution. Based on this mechanism, an ultra-sensitive, cheap and simply practical sensor for detecting glucose and H₂O₂ was developed. Under the most optimal experimental conditions, H₂O₂ and glucose detection results exhibited a good linear range from 0.002 to 3 μM and from 0.02 to 4 μM, respectively, and the detection limits were 1.8 nM and 10 nM, respectively. This approach has been successfully used to detecte glucose in actual biological samples and achieved good results.

Keywords: Hemin, chemiluminescence, luminol, H₂O₂, glucose
Introduction

Chemiluminescence (CL) analysis method, has been widely applied to medical inspection, metal ions analysis, pharmaceutical analysis, life science, environment, material analysis and other fields, because of its unique characteristics of rapid analysis, wide dynamic range, high sensitivity, simple operation and easy automation of the equipment [1–4]. In 1928, Albrecht first reported CL behavior of luminol in alkaline media [5]. As the most commonly used CL reagent at present, luminol was characterized by stable properties, simple structure, good water solubility and high luminous efficiency. The luminol–H₂O₂ CL system still plays a major role in the detection of various substances. Without a suitable catalyst, the chemical reaction is rather slow and the intensity of CL is very weak. Therefore, the main research trends are the discovery of novel catalysts, which can enhance the sensitivity of detection methods and develop new application fields. To date, several kinds of catalysts have been successfully used in the luminol CL system, including transition metal ions, nanomaterials, and biological enzymes [6–8]. As we know, hemin can effectively catalyze the luminol–H₂O₂ CL reaction due to its special structure and biological activity [9]. For example, Luo et al. prepared a metal–organic framework (MOFs) with efficient active sites by embedding hemin in HKUST-1 MOF materials, and established a CL method for detecting glucose, which is based on Hemin@HKUST-1 composite material catalyzed luminol CL reaction [10]. Liu et al. design a hemin-catalyzed luminol–H₂O₂ CL system-based assay for determination of phenol content under alkaline conditions [11].

Hemin is a complex of porphyrin and ferric iron, and has a catalytic center structure similar to cytochrome, peroxidase, myohemoglobin and hemoglobin [12–14]. Hemin has been widely used as a peroxidase enzyme for catalytic oxidation [15]. However, there are relatively limited applications in all fields because hemin is prone to self-polymerize in the catalytic oxidation process and has poor solubility in both organic and aqueous solvents [16]. In order to solve this
problem, the most common method at present is to immobilize hemin on carriers like zeolite, clay, nanoparticles, silica and carbon materials and so on, all of which have a high specific surface area [17]. For instance, Sun et al. reported a novel analytical method, which has been applied in the oxidation or degradation of phenols based on the excellent performance of graphene loaded with hemin to form hybrid nanosheets [18]. Additionally, Liu et al. reported a novel metal-organic framework with catalytic properties obtained by coordination of copper ions and hemin, which successfully achieved detection for H₂O₂ and glucose [16].

In this work, a novel, water-soluble Hemin-mPEG catalyst was synthesized, which is combined and water-soluble porous material mPEG with hemin. The catalyst has proved to be the advantage of not only retained the strong catalytic activity of hemin, but also better water solubility than single hemin. Moreover, Hemin-mPEG represents the generation of catalyst that applied in the luminol–H₂O₂ CL reaction system for the first time, which obviously resulted in a stable slow CL phenomenon. The mechanism of CL-enhancement research indicated the existence of Hemin-mPEG accelerated the generation of 'OH, O₂⁻ and other oxidation radicals. Under the catalysis of Hemin-mPEG, the significantly enhanced CL signal produced by the reaction of glucose oxidation products H₂O₂ with luminol was observed. So, an ultra-sensitive method for detecting glucose and H₂O₂ could be established with Hemin-mPEG as catalyst (Scheme 1). In addition, this work expands the scope of glucose detection methods that can be realized analysis in real samples and adds a new mean to the technology library to fully understand the detection of glucose.

Experimental

Reagents and chemicals

Glucose was obtained from Shantou Xilong Chemical Factory (Guangdong, China). Ascorbic acid was purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Luminol
(3-Aminophthalhydrazide), hemin, poly(ethylene glycol) methyl ether (mPEG, WM2000), nitrotetrazolium blue chloride (NBT), dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), 2,2,6,6-Tetramethylpiperidinoxy (TEMPO), D-lactose, D-mannose, D-xylene and histidine were purchased from Sun Chemical Technology Co., Ltd. (Shanghai, China). Glucose oxidase (GOx), Sodium hydroxide and hydrogen peroxide (30%, \(v/v\)) were purchased from Beijing Chemical Works (Beijing, China). All experimental water involved in this experimentation was ultrapure water and chemical substances were of analytical grade.

Luminol (0.1770 g) was dissolved in NaOH solution (100 mL, 0.1 M) to prepare the 0.1 M luminol stock solution and stored in the dark for at least 15 days. A moderate glucose oxidase (GOx) was dissolved in phosphate buffer solution (pH 7.4) to prepare 1 mg/ml GOx working solution. The \( \text{H}_2\text{O}_2 \) working solution was daily prepared by dilution with deionized water.

**Apparatus**

A BPCL ultra-weak luminescence analyzer (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China) with a photomultiplier voltage of -850v was used to investigate CL experiment in the whole process. Fluorescence spectra were captured by closing the excitation slit on the F-2700 spectrofluorometer (Hitachi, Japan). The UV-3100 UV-VISNIR spectrophotometer (Shimadzu, Japan) was applied to acquire the data of absorption spectra. Infrared spectra were recorded as KBr disks with an Avatar 360 (Nicolet, USA) spectrometer. X-ray photoelectron spectroscopy (XPS) was performed using an ESCALAB 250 spectrometer. The \(^1\text{H} \text{NMR} \) spectrum was obtained under Varian Mercury YH-300 spectrometer operated at 400 MHz.
Preparation for Hemin-mPEG

Water soluble Hemin-mPEG was synthesized on the basis of the Liu’s protocol [19]. Firstly, hemin (0.33 g, 0.5 mmol), mPEG (2.4 g, 1.2 mmol), DCC (0.29 g, 1.4 mmol) and DMAP (0.024 g, 0.2 mmol) were mixed in 20 mL anhydrous methylene chloride and fully stirred at room temperature for 48h, then it was filtered and the filter liquor was evaporated. Finally, the crude product was purified by column chromatography (dichloromethane/methanol = 20/1, v/v). The chromatographic bands of the product were collected, and the solvent was evaporated and dried under vacuum to obtain a dark green solid, Hemin-mPEG. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 5.29 (s, 1H), 3.36 (s, 1H), 3.17 (m, 1H), 2.65 (s, 4H), 1.88 (s, 1H), 1.70 (s, 2H), 1.56 (s, 1H). $^1$H NMR spectrum was shown in Fig. S1.

Procedure of CL measurement

For CL detection of H$_2$O$_2$, 200 $\mu$L 0.5 mM Hemin-mPEG and 200 $\mu$L H$_2$O$_2$ with different concentrations (0, 0.01, 0.25, 1, 2, 4, 10 and 15 $\mu$M) were respectively added to the glass tubes and mixed evenly. After placing the glass tube on the instrument sample holder, 600 $\mu$L 4 mM luminol was quickly injected into the darkroom sample cell via using a static syringe, taking about 4 seconds in the whole process. Meanwhile, the CL kinetic curves were recorded with the BPCL CL analyzer. The relative CL intensity ($\Delta$I = $I_1$ – $I_2$, $I_1$ and $I_2$ represent the CL intensity of the solution with or without H$_2$O$_2$, respectively) was used for quantitative determination.

For CL detection of glucose, 200 $\mu$L different concentrations (0, 0.2, 2, 4, 6, 10, 20 and 40 $\mu$M) of glucose solution and 200 $\mu$L 1mg/mL GOx were mixed and reacted for 10 min at 37 °C. Afterward, 200 $\mu$L mixture was transferred and mixed with Hemin-mPEG (200 $\mu$L of 0.5 mM) in glass tubes. Finally, luminol solution (600 $\mu$L, 4 mM) was rapidly injected, and the CL intensities were detected as described above.
Real serum Sample preparation

Actual samples for glucose detection, clinical serum samples (acquired from The Third Clinical Hospital of Jilin University, Changchun, China) were first treated by centrifugation (10000 rpm, 30 min), then the resulting supernate was diluted 1000 times with PBS buffer solution (pH 7.4) for subsequent measurement.

Results and Discussion

Characterization of Hemin-mPEG

To prove that hemin reacted with mPEG, we especially studied the XPS, IR spectra. XPS analysis was performed to explore the elemental composition of Hemin-mPEG (Fig. 1a). The spectra at 712.8, 531.1, 397.8, and 284.6eV corresponded to Fe 2p, O 1s, N 1s, and C 1s, respectively. Elemental analysis revealed that C, H, N and Fe presence in Hemin-mPEG. The characteristic absorption peaks of the IR spectra of hemin and mPEG are clearly reflected in Hemin-mPEG (Fig. 1b). And from the IR spectrum of Hemin-mPEG, there is a characteristic absorption peak on the carbonyl vibration at 1640 cm⁻¹. At the same time, the IR spectra of Hemin-mPEG showed a stronger methylene (2887 cm⁻¹) and other characteristic absorption peaks of mPEG, which agrees with the results by F. Jun et al [20]. These studies fully demonstrated that mPEG is attached to the hemin.

CL spectra

The CL spectra of the luminol–H₂O₂, luminol–Hemin-mPEG–glucose–GOx, and luminol–Hemin-mPEG–H₂O₂ was recorded in Figure 2. Luminol was oxidized by H₂O₂ will generate a weak CL intensity in the absence of Hemin-mPEG. With the addition of Hemin-mPEG, a strong CL intensity was detected, indicating that the synthetic water-soluble
Hemin-mPEG had excellent catalytic activity in luminol CL reaction. Moreover, the CL strength of luminol–Hemin-mPEG system was significantly increase when a mixture of glucose and GOx was added. Therefore, the newly developed method can also be used to detect glucose. All CL intensity values were recorded at 30s during H$_2$O$_2$ and glucose content testing. The time resolution of CL kinetics measurement was 0.1s.

**Optimization of CL conditions**

The conditions for detecting H$_2$O$_2$ and glucose in the experiment were optimized. Several parameters, such as the concentrations of NaOH, Luminol and Hemin-mPEG, were systematically studied to obtain optimal CL intensity. The effect of different concentrations of Hemin-mPEG in the range of 0.02 – 0.14 mM was studied (Fig. 3a), and the signal of maximum CL was detected at 0.1 mM of Hemin-mPEG. Next, the concentration of Luminol in the system was also examined. As shown in Fig. 3b, when the concentration of Luminol was 2.4 mM, the intensity of CL reached the maximum. Therefore, 0.1 mM of Hemin-mPEG and 2.4 mM luminol were selected as the optimal detection concentration. Finally, the effect of NaOH concentration in the range from 0.05 M to 0.35 M (Fig. 3c) was investigated. To obtain the strongest luminol–H$_2$O$_2$ CL signal intensity, the NaOH concentration was finally chosen to be 0.15M.

**Possible CL mechanism**

In Fig. 4a, the maximum emission peak in luminol–H$_2$O$_2$–Hemin-mPEG CL system is located at 425 nm, which is consistent with the emission wavelength of ultra-weak CL generated by incubating luminol with H$_2$O$_2$. These phenomena indicated that the luminophor of the CL reaction was still the excited state 3-aminophthalate anions (3-APA*). As a result, the Hemin-mPEG was an efficient catalyst to increase the CL intensity. In addition, the UV-visible
absorption spectra with or without Hemin-mPEG in the luminol system were also recorded. In Fig. 4b, the absorption peaks of the luminol and the Hemin-mPEG appeared at 301 nm, 350 nm and 393 nm, respectively. When H$_2$O$_2$ was mixed with the luminol–Hemin-mPEG system, the UV-visible absorption peak was basically unchanged (in Fig 4b), indicating that luminol was not changed and there was no new species generated with adding Hemin-mPEG. From the above discussion, we could draw a conclusion that Hemin-mPEG is just a catalyst in luminol system.

The mechanism of luminol reaction to produce CL has been extensively researched [21]. The three main steps of oxidizing luminol in the solution to produce CL has been shown in Scheme S1. Luminol reacted with hydrogen peroxide in the form of luminol radicals in an alkaline environment. The product was ulteriorly oxidized to form an excited state of 3-aminophthalate anions (3-APA*), which could fleetly change from excited state to ground state with the release of energy in the form of CL [22, 23]. Therefore, the effects of different oxygen-related radicals were discussed in the process of catalyzing luminol–H$_2$O$_2$ CL by Hemin-mPEG catalyst. Ascorbic acid and TEMPO have been generally known to act as broad-spectrum radical scavenger [24]. Adding 4 mM ascorbic acid and TEMPO to the system can effectively inhibit the CL signals of 99.9% and 73.7% respectively, indicating that the production of free radicals occurred during the CL reaction. In addition, specific quenching agents of thiourea, histidine and NBT (hydroxyl radical scavenger, singlet oxygen quencher and superoxide anion scavenger, respectively) were spiked into the solution to detect the influence of these active oxidation radicals on CL system. As can been see from Table 1, the data suggested that the CL signals were inhibited about 48.3%, 89.4% and 13.9% by adding thiourea, NBT and histidine, respectively. Therefore, as crucial intermediates in the system, O$_2$• and OH• generated from H$_2$O$_2$ catalyzed by Hemin-mPEG were participated in CL reaction. As reported in the literature, the intermediate state of the active high-valent metal after oxidation of the metallloporphyrin plays an important role in catalytic process [9, 25]. Hence, according to
literature reports and our experimental results that hemin-mPEG has good catalytic performance to generate $O_2^-$ and 'OH radical from $H_2O_2$, which acts on luminol to produce intense CL.

Analytical figures of merit

The linear response range and detection limit (LOD) of this method for $H_2O_2$ and glucose detection were researched under optimal conditions. In Fig. S2, the relative CL intensity and the $H_2O_2$ concentration exhibited a good linear relationship, ranging from 0.002 to 3 μM. The coefficient of determination was 0.998 and the LOD was 1.8 nM (S/N = 3). This result was better than most published literatures (Table 2).

The $H_2O_2$ generated in the process of the glucose’s oxidation catalyzed by GOx could react with Hemin-mPEG and luminol. Therefore, the designed CL assay can be primely used for the quantitative detecting glucose. The relative CL intensity and glucose concentration within ranging from 0.02 to 4 μM presented a good linear relationship (Fig. 5a). The coefficient of determination was 0.995, in which $C$ is glucose concentration [$10^{-8}$ mol L$^{-1}$]. And the LOD for glucose measured in this experiment was 10 nM (S/N = 3). Furthermore, table 2 contrasted the linear range as well as LOD of $H_2O_2$ and glucose detection using the luminol–$H_2O_2$ CL system between this method and others in previously published literatures. As can be seen, the proposed method has the following distinct advantages: (1) The conditions for the preparation process of catalyst were simple and not harsh, such as high temperature and high pressure (Table S1); (2) The assay we designed possessed high sensitivity and wide detection range.

To evaluate the selectivity of the method, glucose and other glucose analogues, including xylose, lactose and mannose were measured. Fig. 6 showed the signal of the CL had obvious enhancement with addition of glucose, while there were no significant CL signal changes with other glucose analogues. These results revealed that this method we developed has great selectivity to glucose. To assess the value of biosensor in detecting glucose in actual samples,
we tested glucose in human serum samples. The actual sample stock after treatment was selected, and the supernate was diluted with deionized water before the measurement. The determination process of glucose content in the actual sample is the same as that mentioned above in “Procedure of CL measurement”. The concentration of glucose was quantified by CL intensity and linear equation. The results of glucose test based on luminol–H₂O₂–Hemin-mPEG CL system were consistent with those of glucose meter (Table 3). Therefore, the analytical method designed by us can be successfully applied for detecting glucose in actual samples.

Conclusions

In summary, the water-soluble Hemin-mPEG plays a very significant part in catalyzing the luminol–H₂O₂ CL system, resulting in stable and strong CL signals. The mechanism study explained that the luminophor in this CL system being catalyzed by Hemin-mPEG catalyst was still the excited state 3-aminophthalate anions (3-APA*). The reason for the signal enhancement was that Hemin-mPEG exists as catalyst, which promote the production of oxygen-related radicals including 'OH, O₂⁻ and ¹O₂. The proposed CL system was successfully used for detect H₂O₂ and glucose. The method proposed in this study has the advantages of simple, fast, low cost and high selectivity, and demonstrated excellent sensitivity for detecting H₂O₂ as low as 2 nM. This method also accomplishes the detection of glucose in human serum samples, a satisfactory result can be observed by comparing the consistency of detecting glucose with this experiment and the blood glucose meter. The water-soluble Hemin-mPEG which retains the excellent catalytic performance of hemin and overcomes the disadvantages of poor solubility in water, improved the stability of hemin in the catalytic oxidation process, and expanded the application of hemin in the field of CL analysis. In the future, the CL system proposed in this paper will have a potential practical application in pharmaceutical analysis, CL imaging and
immunoassay and so on.

Acknowledgements

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References

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Table 1 Effects of various free–radical scavengers on the luminol–H₂O₂–Hemin-mPEG CL system

<table>
<thead>
<tr>
<th>Quenchers</th>
<th>Intermediates</th>
<th>Concentration</th>
<th>Percent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>·OH, O₂⁻</td>
<td>4mM</td>
<td>99.9%</td>
</tr>
<tr>
<td>TEMPO</td>
<td>·OH, ¹O₂</td>
<td>4mM</td>
<td>73.7%</td>
</tr>
<tr>
<td>Thiourea</td>
<td>·OH</td>
<td>4mM</td>
<td>48.3%</td>
</tr>
<tr>
<td>Histidine</td>
<td>¹O₂</td>
<td>4mM</td>
<td>13.9%</td>
</tr>
<tr>
<td>NBT</td>
<td>O₂⁻</td>
<td>0.4mM</td>
<td>89.4%</td>
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</table>

a. Reaction conditions: H₂O₂ = 0.1 μM, Hemin-mPEG = 0.1 mM, luminol = 2.4 mM in 0.15 M NaOH.

Table 2 Sensitivity of H₂O₂ and glucose detection on the study

<table>
<thead>
<tr>
<th>System</th>
<th>H₂O₂ (µM)</th>
<th>Glucose detection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminol–H₂O₂–Iodophenol blue</td>
<td>0.014</td>
<td>0.025–10</td>
<td>26</td>
</tr>
<tr>
<td>Luminol–H₂O₂–Co–Fe LDHs</td>
<td>0.005</td>
<td>0.010–3</td>
<td>27</td>
</tr>
<tr>
<td>Luminol–H₂O₂–Co₃O₄@SiO₂</td>
<td>0.12</td>
<td>1.00–80</td>
<td>28</td>
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<tr>
<td>Luminol–H₂O₂–Au NCs</td>
<td>0.006</td>
<td>0.020–5</td>
<td>8</td>
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<tr>
<td>Luminol–H₂O₂–CuO NPs</td>
<td>0.011</td>
<td>0.100–5</td>
<td>29</td>
</tr>
<tr>
<td>Luminol–H₂O₂–MIL–53(Fe)</td>
<td>0.05</td>
<td>0.10–10</td>
<td>30</td>
</tr>
<tr>
<td>Luminol–H₂O₂–Hemin–mPEG</td>
<td>0.0018</td>
<td>0.002–3</td>
<td>This work</td>
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Table 3 The analysis results for glucose in human serum samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>CL sensor (mM)</th>
<th>Glucometer (mM)</th>
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<tbody>
<tr>
<td>1</td>
<td>4.40±0.31</td>
<td>4.6</td>
</tr>
<tr>
<td>2</td>
<td>4.91±0.16</td>
<td>4.8</td>
</tr>
<tr>
<td>3</td>
<td>7.36±0.23</td>
<td>7.5</td>
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Figure Captions

Scheme 1. Determination of H₂O₂ and glucose by CL method.

Fig. 1 (a) XPS spectrum of Hemin-mPEG; (b) IR spectra of hemin, Hemin-mPEG and mPEG.

Fig. 2 CL kinetic curves for (a) luminol + H₂O₂, (b) luminol + Hemin–mPEG + glucose + GOx, (c) luminol + Hemin–mPEG + H₂O₂ (6 mM luminol in 0.1 M NaOH, Concentration of H₂O₂ and glucose is 0.4 μM, 40 μM Hemin–mPEG).

Fig. 3 Effect of (a) concentration of Hemin–mPEG (6mM luminol in 0.1 M NaOH solution, 0.4 μM H₂O₂); (b) concentration of luminol (different concentrations of luminol in 0.1 M NaOH solution, 0.4 μM H₂O₂ and 10mM Hemin–mPEG); (c) concentration of NaOH (2.4 mM luminol, 0.4 μM H₂O₂ and 10mM Hemin–mPEG).

Fig. 4 (a) CL spectra of luminol–H₂O₂ and luminol–H₂O₂–Hemin-mPEG (6 mM luminol in 0.1 M NaOH, 40 μM H₂O₂ and Hemin-mPEG); (b) UV-vis absorption spectra of luminol, Hemin-mPEG, luminol–Hemin-mPEG, luminol–H₂O₂–Hemin-mPEG.

Fig. 5 (a) CL spectra detection of different concentrations of glucose; (b) Calibration curve of glucose.

Fig. 6 The selectivity of the CL system for detecting glucose (the concentration of glucose, D-lactose, D-mannose and D-xylose is 0.4 μM).
Scheme 1. Determination of H$_2$O$_2$ and glucose by CL method.

Fig. 1  (a) XPS spectrum of Hemin-mPEG; (b) IR spectra of hemin, Hemin-mPEG and mPEG.
Fig. 2  CL kinetic curves for (a) luminol + H₂O₂, (b) luminol + Hemin–mPEG + glucose + GOx, (c) luminol + Hemin–mPEG + H₂O₂ (6 mM luminol in 0.1 M NaOH, Concentration of H₂O₂ and glucose is 0.4 μM, 40 μM Hemin–mPEG).
Fig. 3  Effect of (a) concentration of Hemin–mPEG (6mM luminol in 0.1 M NaOH solution, 0.4 μM H₂O₂); (b) concentration of luminol (different concentrations of luminol in 0.1 M NaOH solution, 0.4 μM H₂O₂ and 10mM Hemin–mPEG); (c) concentration of NaOH (2.4 mM luminol, 0.4 μM H₂O₂ and 10mM Hemin–mPEG).
Fig. 4  (a) CL spectra of luminol–H$_2$O$_2$ and luminol–H$_2$O$_2$–Hemin-mPEG (6 mM luminol in 0.1 M NaOH, 40 μM H$_2$O$_2$ and Hemin-mPEG); (b) UV-vis absorption spectra of luminol, Hemin-mPEG, luminol–Hemin-mPEG, luminol–H$_2$O$_2$–Hemin-mPEG.

![Diagram of CL spectra and UV-vis absorption spectra](image)

Fig. 5  (a) CL spectra detection of different concentrations of glucose; (b) Calibration curve of glucose.

![Diagram of CL spectra detection and calibration curve](image)
Fig. 6 The selectivity of the CL system for detecting glucose (the concentration of glucose, D-lactose, D-mannose and D-xylose is 0.4 μM).