Sensitive and Selective Carmine Acid Detection Based on Chemiluminescence Quenching of Layer Doubled Hydroxide–Luminol–H$_2$O$_2$ System

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Supporting Information

ABSTRACT: Carminic acid (CA) extracted from cochineal is widely used in food additives as a natural colorant, and its potential risk to human health makes its detection important. In this work, a layered doubled hydroxide (LDH)–luminol–H$_2$O$_2$ system-based chemiluminescence (CL) platform has been successfully applied for CA sensing. The principle detection consists of two steps: first, LDH adsorbs CA onto the surface via electrostatic attraction; second, CA quenches the CL of the LDH–luminol–H$_2$O$_2$ system via the synergistic effect of CL resonance energy transfer, reduction of reactive oxygen species, and occupation of positively charged centers of brucite-like layers. With this CL approach, 0.5 μM CA is detectable using a CL spectrometer, and the limit of detection is 0.03 μM. This CL system exhibited a linear response to CA in the concentration range from 0.5 to 10 μM. In addition, the practical application of the designed CL sensing system is evaluated with dried pork slice samples.

INTRODUCTION

Carminic acid (CA), an anthraquinone glycoside from dried cochineal with high solubility due to its sugar and carboxyl group residues, has been widely used as red colorant in food or beverage to make them colorful and in influence the consumer’s choice.1 Despite its relatively high chemical and biological stability, the use of it as a colorant or additive is absolutely controlled by laws and regulations because of its potential risk to human health.2 The allowable concentration range of CA in foodstuffs is set to be 25–500 mg/kg by the Standardization Administration of China (GB 2760-2014). Thus, the exploration of a simple and rapid CA detection method is important to understand its biological function and prevent its contamination. Toward this goal, many CA detection approaches based on high-performance liquid chromatography, voltammetry, mass spectrometry, and spectrophotometry have been reported.3–8 Among them, spectrophotometric detection with easy operation and rapid response has attracted growing attention.6,9 However, the drawback of spectral overlapping generally diminishes the resolution and accuracy of these methods. Therefore, it is of great significance to develop a new spectrophotometric detection method with high sensitivity and accuracy.

As one of the frequently used spectrophotometric techniques, chemiluminescence (CL) spectrophotometry has been widely used in the analytical-related fields in recent years because of its characters of low background noise, fast response, free excitation source, and simple instrumentation.10–13 The sensitivity of CL-based techniques is sometimes inhibited by its low emission intensity. Interestingly, the nanomaterial-improved novel CL systems with satisfying sensitivity have been widely investigated because of the ever-increasing nanotechnology.14–20 For example, with the introduction of gold nanoparticles, the CL signal of the luminol–H$_2$O$_2$ system is greatly enhanced.16,21 Similarly, upon the addition of layered double hydroxide (LDH), a 950-fold enhancement of the CL signal of the luminol–H$_2$O$_2$ system is found.22 The sensitivity of H$_2$O$_2$ detection using a luminol CL system is increased by 2 orders of magnitude with the assistance of LDH.23 By combining flow system and injection detector, sensitive CL detection systems have been established for dyes, thiols, H$_2$O$_2$, and so forth.24,25 With the integration of the electrochemical technique, sensitive peptide detection and enzyme activity evaluation are achieved.26,27 The high sensitivity and the excellent characters encourage us to develop a simple CL approach for CA detection.

In this study, a facile and rapid method for sensitive and accurate CA detection based on the LDH–luminol–H$_2$O$_2$ CL
system was reported. The detection mechanism is assigned to the synergistic effect of CL resonance energy transfer (CRET), reduction of reactive oxygen species (ROS), and occupation of positively charged centers of brucite-like layers by the anthraquinone motif, which suppress the CL signal. The CL quenching effect of structural motif CA was also systematically investigated for the first time. In addition, the strategy shows high selectivity toward CA over other small molecules, anions, and metal ions. Moreover, the simple CA sensing approach exhibits satisfying sensitivity toward CA with a limit of detection of 0.03 μM and shows a linear response in the concentration range from 0.5 to 10 μM. Furthermore, the practical application of the proposed method was validated by detecting CA detection in dried pork slice samples.

### RESULTS AND DISCUSSION

**CA-Induced CL Quenching of the LDH–Luminol–H₂O₂ System.** At the starting point, the CL response of the LDH–luminol–H₂O₂ system to CA was investigated. As shown in Figure 1, the CL signal of the luminol–H₂O₂ system was dramatically enhanced by LDH. The LDH-enhanced CL is attributed to the LDH-promoted adsorption of the peroxide anion and luminol dianion, which facilitates the formation of carbonate radicals and luminol radicals and is demonstrated in previous reports.²³ The promoted CL intensity of the LDH–luminol–H₂O₂ system, however, was dramatically inhibited with the addition of CA, suggesting that CA possesses a high quenching efficiency to this CL system.

**Structure-Mediated CL Quenching.** For most of the quencher studies, only energy or electron transfer between the whole molecule and luminescence system is considered. However, the contribution of the inside structural motif is rarely investigated. These motifs, however, play important roles on the optical and/or electronic characters of quenchers. In this study, the CL quenching effect of the structural motif of CA was systematically studied. The molecular structure of CA consists of an anthraquinone, a sugar residue, and a carboxyl group (Figure 2). The structure and the carboxyl group do not cause CL inhibition according to previous studies.²⁴ In consideration of the structural character of CA, the anthraquinone structure is probably responsible for the CL quenching. To verify this hypothesis, the effects of five different anthraquinone analogues (Figure S1) including alizarin (1,2-DHAQ), alizarin red S, alizarin cyanin green F (ACGF), anthraquinone-2-sulfonic acid (ASA), and anthraquinone-1,8-disulphonic acid (ADA) on the CL profile were studied. As showed in Figure S2, all five analogues showed CL quenching capabilities to the LDH–luminol–H₂O₂ system, indicating that anthraquinone indeed is the possible structure responsible for CL quenching.

As reported in our previous work, the LDH–luminol–H₂O₂ system shows a CL emission peak around 425 nm.²² As shown in Figure S3, both 1,2-DHAQ and ACGF showed an absorption peak around 425 nm, which is overlapped with the CL emission spectra of excited luminol. The absorption spectra of ASA and ADA, however, have no overlap with the CL emission spectra of excited luminol. They exhibited only slight response to the CL profile of the LDH–H₂O₂–luminol system. These results suggest that the energy transfer between 3-aminophthalate anions and anthraquinone analogues might cause the CL quenching. However, ACGF also showed a visible absorption peak around 430 nm, which provides an overlap with the CL emission spectra of excited luminol. The CL quenching capability is very low, indicating that the energy transfer is not the only way that leads to CL quenching. In addition, 1,2-DHAQ and ACGF, with similar absorption profiles, showed totally different CL quenching capabilities, further suggesting the importance of the structural motif to the CL quenching performance.

Notice that 1,2-DHAQ exhibited the highest CL quenching efficiency over other four compounds, suggesting that the substituent group on anthraquinone may also be essential to the CL quenching ability. Thus, the CL quenching effects of various anthraquinone compounds with different substituent groups should be investigated. According to Figure S2, anthraquinone compounds with only sulfate group showed weak quenching abilities, implying that the CL inhibition is not caused by the sulfate group. In contrast, the group on the benzene ring largely promotes the CL quenching capability. In this work, six anthraquinone compounds with different substituent groups (hydroxyl group) were studied, including 2-hydroxy anthraquinone (2-HAQ), 1-hydroxy anthraquinone (1-HAQ), 1,5-dihydroxyanthraquinone (1,5-DHAQ), quinizarin (1,4-DHAQ), 1,2-DHAQ, and purpurin (1,2,4-THAQ).

According to Figure 3, the CL quenching capability is generally increased with the increasing hydroxyl group number. Meanwhile, the position of the substituent hydroxyl group also affects the CL quenching capability. These results proved that the substituent hydroxyl group is important to the CL quenching capability of anthraquinone compounds. In other words, hydroxyanthraquinone possesses high CL quenching capability.

To realize the universality of this substituent hydroxyl group-promoted CL quenching capability, hydroxyanthraquinone-mediated CL quenching of other two conventional systems, including LDH–H₂O₂–lucigenin system and LDH–H₂O₂–NaIO₄ system were studied. As shown in Figure 4, in all three CL systems, 1,2,4-THAQ showed the highest CL quenching capability.
planes of (003), (006), and (110). Also, the asymmetric LDH showed symmetric and sharp re

pounds were performed. According to Figure 5a, the prepared (FT-IR), and surface charge characterizations of the LDH in the presence of various hyanthraquinone compounds. From I to VII: only LDH, 2-HAQ-absorbed LDH, 1-HAQ-absorbed LDH, 1,5-DHAQ-absorbed LDH, 1,4-DHAQ-absorbed LDH, 1,2-DHAQ-absorbed LDH, and 1,2,4-THAQ-absorbed LDH, respectively.

Quenching capability. In addition, the trends of the substituent hydroxyl group-promoted CL quenching efficiency in the LDH–H2O2–lucigenin system and the LDH–H2O2–NaIO4 system were consistent with that in the LDH–H2O2–luminol system. All these results suggest that 1,2,4-THAQ motif is responsible for the high CL quenching efficiency of CA.

Mechanism of Hydroxanthraquinone-Promoted CL Quenching. It was found that negligible CL quenching induced by hydroxanthraquinone compounds was observed without LDH, suggesting LDH is important to get the hydroxanthraquinone compounds and H2O2–luminol system close and assist the CL quenching. To first understand the interaction between hydroxanthraquinone compounds and LDH, X-ray diffraction (XRD), Fourier-transform infrared (FT-IR), and surface charge characterizations of the LDH in the absence and presence of hydroxanthraquinone compounds were performed. According to Figure 5a, the prepared LDH showed symmetric and sharp reflections for characteristic planes of (003), (006), and (110). Also, the asymmetric reflections for (012), (015), and (018) planes were observed. These characteristic reflections indicate the existence of hydrotalcite-like structure.25,30 The addition of other hydroxanthraquinone compounds, however, did not change the XRD patterns of LDH. The remaining symmetric and asymmetric reflections revealed the unchanged hydrotalcite-like structure, suggesting that those hydroxanthraquinone compounds were not inserted into the interlayer of LDH. Because the introduction of LDH greatly enhanced the CL quenching capability of hydroxanthraquinone compounds, approaching these compounds to the CL system in the presence of LDH is necessary. Therefore, adsorption of these compounds onto the LDH surface might be the possible pathway to get them close. Such a hypothesis was first proved by FT-IR characterization of LDH upon adding different hydroxanthraquinone compounds. The corresponding FT-IR spectra are shown in Figure 5b. The characteristic peaks around 3491, 1660, 1383, and 665 cm−1 belong to the stretching vibration of the hydroxyl groups, flexural vibration of hydroxyl in H2O, asymmetric vibration of intercalated CO3−, and metal–oxygen lattice vibration, indicating the preservation of the hydrotalcite-like structure.31 Accordingly, peaks around 1270 and 1560 cm−1 that are assigned to the vibration of the anthraquinone ring appeared when hydroxanthraquinone compounds were added, suggesting the adsorption of these hydroxanthraquinone compounds onto the surface of LDH.32,33 The adsorption of hydroxanthraquinone compounds was further verified by zeta potential measurements. As shown in Figure 5c, the surface charge of LDH was determined to be +43.5 mV, which is consistent with previous reports.34 High surface charge benefits the adsorption of these compounds onto its surface. As expected, it decreased over 25% after the addition of 100 μM hydroxanthraquinone compounds. Moreover, it can be seen that the hydrodynamic diameter of LDH showed only a slight variation if 100 μM hydroxanthraquinone compounds were added (Figure 5d), suggesting that the addition of these compounds do not cause the aggregation of LDH. This result also demonstrated that only surface adsorption of hydroxanthraquinone compounds happens.

It is generally accepted that the CL emission of the LDH–H2O2–luminol system can be inhibited by some small organic molecules or inorganic fluorophores through different mechanisms, including CRET, reduction of generated ROS, or occupation of positively charged centers of brucite-like layers.15 The latter two mechanisms inhibit the generation of light-emitting intermediate 3-aminophthalate anions, whereas the former one only transfers the energy of the intermediate.
To figure out the CL quenching mechanism, the absorption spectra of these hydroxyanthraquinone compounds were measured. As shown in Figure S4, 1,2,4-THAQ and 1,4-DHAQ with distinct absorption around 460–490 nm displayed high CL quenching efficiencies. The maximum emission wavelength of the intermediate 3-aminophthalate anion is nearly 425 nm, suggesting that the CRET process may exist. However, 1,5-DHAQ with weak absorption around 430 nm also showed high CL quenching capability. Also, 1-HAQ with high adsorption around 420 nm only showed weak CL quenching ability. The different CL quenching behaviors of them may be due to the various quantum yields. The quantum yields of all six hydroxyanthraquinone compounds were also determined. The diverse quantum yields may contribute differently to the CRET and lead to different CL quenching capabilities (Figure S5a–c). These results indicate that the CRET is not the only way to cause CL quenching. Notice that the phenol group possesses strong reducibility, and the interaction between hydroxyanthraquinone and hydroxyl radical (•OH) may also weaken the CL signal. To verify the influence of the phenol group, the reducibilities of hydroxyanthraquinone compounds were investigated by using 7-hydroxycoumarin-3-carboxylic acid (OHCCA) as the reporter. It is reported that coumarin-3-carboxylic acid (3-CCA) can be oxidized by •OH to form fluorescent OHCCA. With the addition of hydroxyanthraquinone compounds, the fluorescence of OHCCA decreased (Figure S6). It was seen that 1,2,4-THAQ with strong CL inhibition capability showed high reducibility (Figure S5d), however, 1-HAQ with weak CL quenching capability also showed high reducibility. Also, 1,4-DHAQ and 2-HAQ with similar reducibilities possess totally different CL quenching capabilities. In this case, reducibility of hydroxyanthraquinone compounds play an important but not the only role on CL quenching. In addition, the surface adsorption of hydroxyanthraquinone compounds is also essential to CL quenching. As mentioned above, the zeta potential of LDH dramatically decreased after adding hydroxyanthraquinone compounds, indicating that the occupation of positively charged centers of brucite-like layers is also important to CL quenching. It should be noticed that only small differences in the zeta potentials of hydroxyanthraquinone compounds-functionalized LDH were observed. The small differences of zeta potential imply that the CL quenching capability is partially related to the occupation of positively charged centers of brucite-like layers. Therefore, we suppose that the synergy of CRET, reduction of ROS, and occupation of positively charged centers of brucite-like layers lead to the strong CL quenching capability of hydroxyanthraquinone compounds. Taken together, the CA-caused CL quenching consists of two steps: first, LDH adsorbs it onto the surface via electrostatic attraction; second, CA quenches the CL signal through the synergy of CRET, reduction of ROS, and occupation of positively charged centers of brucite-like layers. The proposed mechanism is shown in Figure 6.

**Sensitivity and Selectivity of CA Sensing System.** Although these hydroxyanthraquinone compounds showed different CL quenching capabilities, interestingly, they did not cause any evident change in the CL emission profile. As shown in Figure S7, the addition of hydroxyanthraquinone compounds leads to the decrease of CL intensity but without change of CL emission spectra. In addition, the CL kinetics of LDH–H₂O₂–luminol system showed a negligible variation upon adding hydroxyanthraquinone compounds. These results suggest that addition of CA only induces the diminution of CL intensity. In other words, the LDH–H₂O₂–luminol system can act as a CL sensing platform for CA. The CL signal of the LDH–H₂O₂–luminol system is strong under alkaline conditions, and our previous studies also indicate that the optimal pH of CL quenching induced by other targets is almost near pH 9.5; we thus performed all subsequent sensing experiments at pH 9.5. The concentration of LDH at 30 mg/mL displays the maximum CL quenching efficiency based on our previous study, which was used in the following experiments.

Upon increasing the CA concentration, the CL signal of the LDH–H₂O₂–luminol system gradually decreased, as shown in Figure 7. The CL intensity showed a good linear relationship

\[ \text{CL} = K \times [Q] + I_{\text{CA}} \]

(R² = 0.991) versus CA concentration ranging from 0.5 to 10 μM and is easily described by the linear equation, \( I_{\text{CL}} = K[Q] + 4182.9 \), where \( I_{\text{CA}} \) is the CL intensity, slope K is the CL quenching constant, and \([Q]\) stands for the CA concentration. The CL quenching constant was calculated to be \(-3.25 \times 10^8\) M⁻¹ by linear regression of the plot. The detection limit toward CA was determined to be 0.03 μM (S/N = 3), which is more sensitive than other reported methods. The relative standard deviation obtained from 11 times repeated measurements of 2 μM CA was 2.9% (Figure S8), indicating the high accuracy of CA detection using this proposed method.

To verify whether the CA-induced CL quenching is specific, the CL signals were recorded with the addition of various small molecules, metal ions and anions, including citric acid, sucrose, glucose, sodium benzoate, HPO₄²⁻, SO₄²⁻, Mg²⁺, Al³⁺, Ca²⁺, Zn²⁺, Ba²⁺, K⁺, NH₄⁺, Na⁺, Cl⁻, and NO₃⁻. Although 1,2-DHAQ can also induce CL inhibition, it was not considered in
the specificity evaluation in this work. It is known that 1,2-DHAQ is generally used as a colorant in cotton products. However, it cannot be used as a food colorant because of its high toxicity. In this case, we just investigated several possible chemicals in the dried pork product to evaluate the specificity of our proposed CL detection system. As indicated in Figure 8a, none of these small molecules, metal ions, or anions (100 μM) could induce a conspicuous CL decrease as CA (5 μM) did. In addition, neither small molecules nor ions would interfere in the detection of CA (Figure 8b), revealing excellent selectivity of the assay toward CA. Despite the negative charge, the high concentration Cl⁻ or NO₃⁻ did not show any suppression effect on the CL signal. One possible reason is the smaller size of these anions compared to that of CA. Thus, the adsorption of the anion has just a slight effect on the capture of the luminol dianion and peroxide anion. The high tolerance concentrations of interfering molecules or ions suggest that this method is highly specific even under a high concentration of interferents. Compared to other reported works, this proposed method provides better or comparable selectivity toward CA over other possible interferents. Therefore, it could be concluded that the proposed method showed high selectivity and sensitivity toward CA.

CA Analysis in Dried Pork Slice Samples. To evaluate the applicability and reliability of the proposed method in real sample, we tested CA in treated dried pork slice samples. Dried pork slice was phased from the supermarket without treatment. CA was extracted from the slice using a strand method. The CL signal of the LDH–H₂O₂–luminol system was suppressed after the addition of real sample extraction (Figure S9), indicating the existence of carmine acid in dried pork slice samples. The actual concentration of carmine acid was determined to be 4.36 μM by a standard addition approach (Table S1), which is much lower than the allowable concentration. To further verify the accuracy of carmine acid detection in dried pork slice samples, a current standard method for carmine acid detection based on ultraviolet–visible (UV–vis) spectrophotometry was performed. The detected concentration of carmine acid was 4.02 μM. The low CA content indicates that the dried pork slice sample from the supermarket meets the demand of food criterion. The comparable results suggest that our method is capable for carmine acid detection in a real sample. Moreover, the high recoveries of carmine acid determination in the dried pork slice sample ranging from 97.3 to 99.8% also proved the practical application of the proposed method. In a word, the proposed method is feasible for detecting carmine acid in real samples.

### CONCLUSIONS

In summary, we have reported a facile CL platform to detect carmine acid based on the LDH–luminol–H₂O₂ system. Highly specific CA detection is achieved through the surface adsorption and synergistic effect of CRET, reduction of ROS, and occupation of positively charged centers of brucite-like layers, which provide the CL sensor with satisfying interference rejection of over 20 folds than other small molecules, anions, and metal ions. This approach shows high sensitivity toward CA with a limit of detection of 0.03 μM. Our study also demonstrates an interesting combination of electrostatic attraction and multiplex CL quenching mechanisms; thus, new avenues for the design of luminol–H₂O₂ CL nanosensors for other analytes based on a similar strategy might open up in the analytical and related fields.

### EXPERIMENTAL SECTION

#### Chemicals.
Anhydrous sodium carbonate (Na₂CO₃), sodium hydroxide (NaOH), sodium sulfate (Na₂SO₄), sodium phosphate (Na₃PO₄), anhydrous sodium dihydrogen phosphate (NaH₂PO₄), sodium chloride (NaCl), sodium benzoate, magnesium nitrate hexahydrate (Mg(NO₃)₂·6H₂O), zinc nitrate hexahydrate (Zn(NO₃)₂·6H₂O), calcium nitrate tetrahydrate (Ca(NO₃)₂·4H₂O), potassium chloride (KCl), potassium nitrate (KNO₃), aluminum nitrate nonahydrate (Al(NO₃)₃·9H₂O), barium chloride dihydrate (BaCl₂·2H₂O), and potassium nitrate (KNO₃) were purchased from Xilong Scientific Co., Ltd. (Guangzhou, China). Sodium periodate (NaIO₄) and ammonium nitrate (NH₄NO₃) were purchased from Tianjin Fuchen Chemical Reagents Factory (Tianjin, China). 1-HAQ and 1,4-DHAQ were selected from Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). 1,5-DHAQ was obtained from Alfa Aesar Chemicals Co., Ltd. (Shanghai, China). 1,2-DHAQ, anthraquinone-2-sulfonic acid, and lucigenin were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). ACGF, ADA, and CA were selected from TCI. (Shanghai, China). 3-CCA was obtained from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, USA). Luminol was purchased from J&K Chemical Ltd. (Beijing, China). Working solution of H₂O₂ was daily prepared by diluting the 30% stock solution of H₂O₂ with deionized water. Luminol stock solution (0.01 M) was prepared by diluting 0.1 M luminol NaOH solution and was used after 14 days storage. The working luminol solution was obtained by diluting the stock solution with deionized water.
periodate stock solution and their corresponding working solutions were prepared in a similar way. All reagents used were of analytical grade and used as received without any further purification. Deionized water (18.2 MΩ) was obtained from a Millipore system (Milli-Q, Millipore).

**Apparatus.** Powder XRD measurements were collected with a Bruker D8 ADVANCE X-ray diffractometer (Bruker AXS GmbH, Germany) equipped with graphite-monochromatized Cu/Kα radiation (λ = 1.5406 Å). The 2θ angle of the diffractometer was stepped from 5° to 70° at a scan rate of 10°/min. FT-IR spectra were recorded with a Nicolet 6700 FT-IR spectrometer (Thermo, American). Zeta potential and hydrodynamic diameter were measured by using a Zetasizer 3000HS nanogranularity analyzer (Malvern Instruments). Absorption spectra were obtained by a UV-3900 UV–vis spectrophotometer (Hitachi, Japan). The CL signal was measured on an ultraweak biophysics CL (BPCL) analyzer (Institute of Biophysics, Chinese Academy of Science, Beijing, China). The CL spectrum of this system was measured using a F-7000 fluorescence spectrophotometer with the assistance of high-energy cutoff filters from 400 to 540 nm (Hitachi, Japan). The fluorescence spectra were performed using a F-7000 fluorescence spectrophotometer (Hitachi, Japan). The slits of emission and excitation are 5.0 nm, the voltage is 700 V, and the scanning rate is 1200 nm/min. The quantum yields were measured with a FLS980 transient steady-state fluorescence spectrometer (Edinburgh, U.K.).

**Synthesis of LDHs.** The Mg–Al–CO₃ LDHs were synthesized by coprecipitation methods. The precipitation process was carried out under low supersaturation conditions at a constant pH. For an Mg/Al molar ratio of 3, solution A process was carried out under low supersaturation conditions synthesized by coprecipitation methods. The precipitation (photomultimeter) PMT. Then, 100 emission spectra were measured through a F-7000 compounds were measured using the same procedure. The CL data acquisition. CL signals from other hydroxyanthraquinone were monitored by PMT and imported to the computer for detection of CA in the dried pork slice sample, and schematic diagram of the CL detection system (PDF).

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