Ruthenium Ion-Complexed Carbon Nitride Nanosheets with Peroxidase-like Activity as a Ratiometric Fluorescence Probe for the Detection of Hydrogen Peroxide and Glucose

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ABSTRACT: Detection of hydrogen peroxide is of great significance for clinical diagnosis and biomedical research. Ratiometric detection represents an effective method that is generally based on horseradish peroxidase. In the present study, ruthenium ion-complexed carbon nitride (Ru–C₃N₄) nanosheets are found to serve as a peroxidase mimic and can catalyze the conversion of o-phenylenediamine to fluorescent 2,3-diaminophenazine in the presence of H₂O₂. The produced 2,3-diaminophenazine also results in the apparent quenching of the Ru–C₃N₄ photoluminescence due to the inner filter effect. These unique characteristics can be exploited for the construction of an effective, peroxidase-free ratiometric fluorescence framework for the detection of H₂O₂ and glucose, which has also been used in the successful detection of glucose in human serum. Results from this study not only demonstrate a new peroxidase mimic but also provide a novel ratiometric fluorescence platform for the detection of H₂O₂ and metabolites involving reactions of H₂O₂ generation in the absence of horseradish peroxidase.

KEYWORDS: ion complexation, carbon nitride, peroxidase mimic, ratiometric fluorescence probe, hydrogen peroxide, glucose

INTRODUCTION

Hydrogen peroxide (H₂O₂) is a critical metabolite in cells and plays a vital role in physiological health. For instance, H₂O₂ is a signal molecule related to oxidative stress and physiological activity. An abnormal level of H₂O₂ can increase the risk of central nervous system diseases and cancers. Therefore, the detection of H₂O₂ is of great significance for biomedical research and clinical diagnosis. Thus far, a range of techniques have been reported for H₂O₂ detection, including electrochemistry, colorimetry, fluorescence, and chemiluminescence. Of these, the fluorescence method shows many advantages, such as rapid analysis, good sensitivity, and high selectivity. Compared with single fluorescence measurement, ratiometric fluorescence measurements can minimize erroneous signals from environmental influences and exhibit improved sensitivity and precision, because it is based on the concurrent recording of two fluorescence signals at a single excitation wavelength. For instance, ratiometric fluorescence detection of H₂O₂ has been reported by using horseradish peroxidase (HRP) as the enzymatic catalyst. In one study, HRP catalyzes the oxidation of nonfluorescent o-phenylenediamine (OPD) to fluorescent 2,3-diaminophenazine (DAP), and DAP quenches the fluorescence of graphitic carbon nitride (C₃N₄). These observations can be exploited for the construction of a ratiometric fluorescence platform for the detection of H₂O₂ based on the decrease of the C₃N₄ fluorescence and the enhancement of the DAP fluorescence. However, reports of ratiometric fluorescence detection of H₂O₂ in the absence of HRP have been scarce. Notably, the use of peroxidase mimetics as the fluorescence probe may enable the detection of H₂O₂ in the absence of HRP, which will be useful for living cell analysis in the future.

Enzyme mimetics hold great potential in replacing natural enzymes because of their low costs and high stability. In 2007, magnetic Fe₃O₄ nanoparticles were found to behave as peroxidase mimics. Since then, a vast variety of (nano)-materials also exhibit peroxidase-like activities. These include noble metals, metal oxides, metal complexes, and carbon materials. C₃N₄ and metal nanoparticles/C₃N₄ composite materials have also been exploited as peroxidase mimetics for the colorimetric detection of H₂O₂. However, ratiometric fluorescence detection of H₂O₂ has not been achieved based on peroxidase mimetics, which is anticipated to exhibit higher sensitivity and better selectivity than conventional colorimetric detection. This is the main motivation of the present work.

In an earlier study, we demonstrated that the abundant pyridinic nitrogen of carbon nitride nanosheets could be...
exploited for the complexation of ruthenium(II) ions (Ru–C₃N₄), which exhibited high electrocatalytic activity toward hydrogen evolution reaction. In the present study, it was found that Ru–C₃N₄ also shows high peroxidase-like activity and might be exploited for the construction of a novel, effective ratiometric fluorescence platform for the detection of H₂O₂ (Scheme 1). Experimentally, OPD was oxidized by H₂O₂ to produce DAP in the presence of Ru–C₃N₄ (in place of HRP) that exhibited a fluorescence emission at 565 nm, and concurrently the fluorescence emission of Ru–C₃N₄ (at 455 nm) was quenched by the generated DAP due to the inner filter effect. The diminishment of the Ru–C₃N₄ fluorescence together with the enhancement of the DAP fluorescence enables the ratiometric fluorescence detection of H₂O₂. To the best of our knowledge, this is the first demonstration of peroxidase-like activity by Ru–C₃N₄ nanocomposites and its use as a peroxidase-free ratiometric fluorescence probe for the detection of H₂O₂. The detection limit for H₂O₂ (50 nM) is amongst the lowest ones reported previously, highlighting an effective strategy for the sensitive detection of H₂O₂. The unique property is also exploited for the sensitive and selective detection of glucose, which is known to generate GOx.

**EXPERIMENTAL SECTION**

**Chemicals.** Glucose oxidase (GOx; 150 kU g⁻¹) was obtained from Sigma-Aldrich. Melamine, o-phenylenediamine (OPD), and hydrogen peroxide (H₂O₂; 30 wt%) were purchased from Sinopharm Chemical Reagents Co. Ltd. Ruthenium(III) chloride (RuCl₃), glucose, maltose, sucrose, galactose, bovine serum albumin (BSA), ascorbic acid, uric acid, and amino acids were purchased from Aladdin Chemicals Co., Ltd. Human serum samples were obtained from healthy volunteers in the Fourth Hospital of Changsha. All other reagents were of analytical or better grade and used as received. Acetate buffer solution was prepared with 0.1 M acetic acid and sodium acetate (pH 4.5). Phosphate buffer solution (PBS, pH 7.0) was prepared with 0.1 M KH₂PO₄ and Na₂HPO₄. Water was purified with a Millipore Milli-Q System (resistivity 18.3 MΩ·cm).

**Preparation of Ru–C₃N₄.** The preparation of Ru–C₃N₄ has been detailed previously. Experimentally, graphitic C₃N₄ nanosheets were first produced by the thermal treatment of melamine in air followed by sonication in water:⁶,⁷ (i) a melamine (10 g) was loaded into a ceramic crucible with a cover, which was put into in a muffle furnace and heated at 600 °C for 3 h at the heating rate of 2.3 °C min⁻¹, and (ii) 50 mg of the above products were dispersed into 50 mL of H₂O, and C₃N₄ thin layers were formed by sonication overnight.

Ru–C₃N₄ was then prepared by thermal refluxing of the obtained C₃N₄ and RuCl₃ in water.⁶ In brief, 56 mg of RuCl₃ was added to the above C₃N₄ dispersion, and the mixture was refluxed for 4 h. Centrifugation was then used to separate the product, and excess ruthenium ions were removed by washing the collected solids with water and ethanol.

Characterization. High-resolution transmission electron microscopy (TEM) images were acquired with a Tecnai G2 F-30 transmission electron microscope. X-ray photoelectron spectroscopy (XPS) studies were carried out with a Thermo Fisher XPS instrument. Ultraviolet–visible (UV–vis) absorption spectra were collected with a Shimadzu UV-2450 spectrophotometer. Fluorescence spectroscopic studies were performed on a Hitachi F-7000 spectrophotometer. An Agilent 1200-7700 quantum reaction spectrometer was used for inductively coupled plasma mass spectrometric (ICP-MS) analysis. Zeta potentials were evaluated with a Malvern Zetasizer Nano Z Analyzer.

**Detection of H₂O₂.** 10 μL of a H₂O₂ aqueous solution at different concentrations was added into 1 mL of the 0.10 M acetate buffer solution (pH 4.5) containing 50 μg mL⁻¹ Ru–C₃N₄ and 0.2 mM OPD. After incubation at 25 °C for 10 min, fluorescence measurements were carried out with the excitation set at 370 nm.

**Detection of Glucose.** Experimentally, a 10 μL solution containing 0.10 M PBS (pH 7.0), 0.50 mg mL⁻¹ GOx and glucose of different concentrations was first prepared. After incubation at 25 °C for 30 min, the solution was transferred into a 1 mL 0.10 M acetate buffer solution (pH 4.5), which contained 50 μg mL⁻¹ Ru–C₃N₄ and 0.2 mM OPD. Additional incubation was allowed for 10 min at 25 °C, before fluorescence spectra were acquired at 370 nm excitation. To detect glucose in human serum, the sample was diluted with PBS by a factor of 20, before fluorescence spectra were collected.

**RESULTS AND DISCUSSION**

Ru–C₃N₄ was prepared by the thermal refluxing of graphitic C₃N₄ and RuCl₃ in aqueous solution, where the ruthenium metal centers were incorporated within the C₃N₄ scaffold by coordination to two pyridinic nitrogen moieties (RuN₂), as described previously (Scheme 1).⁶ The morphology of Ru–C₃N₄ was first characterized by TEM measurements. As shown in Figure 1a,b, Ru–C₃N₄ shows a nanosheet structure of a few tens of nanometers across, similar to that of pristine C₃N₄ (Figure S1a). Figure 1c shows the high-angle annular dark-field scanning TEM (HAADF-STEM) image of Ru–C₃N₄ and elemental maps based on energy-dispersive X-ray (EDX) analysis, where the C, N, Ru, and Cl elements can be seen to be uniformly distributed in the sample. Not that no crystalline Ru species was found in Ru–C₃N₄, as demonstrated in high-resolution TEM and X-ray diffraction studies. ICP-MS measurements showed that the Ru content in Ru–C₃N₄ was ca. 9.97 atom %.

The chemical composition of Ru–C₃N₄ was further examined by XPS measurements. From the survey spectrum in Figure 1d, one can readily identify the C, N, Ru, and Cl elements. Figure 1e shows the corresponding high-resolution spectrum of the Ru 3d and C 1s electrons. The Ru 3d₅/₂ and 3d₃/₂ electrons can be deconvoluted at 281.3 and 285.2 eV, which are consistent with those of Ru(II) in ruthenium bipyridine complexes,⁵⁵ suggesting that the Ru centers were mainly in the +2 valence state, as detailed previously.⁵⁶ Two C 1s subpeaks can be found at 284.5 and 288.0 eV, which can be assigned to the defective C in sp³ C–C and sp²-hybridized C in N–C≡N, respectively. Notably, the latter binding energy (288.0 eV for C in N–C≡N) shows a marked blue shift for Ru–C₃N₄ as compared to that (287.3 eV) of pristine C₃N₄ (Figure S1b), due to the formation of Ru–N coordination bonds.⁶ Two nitrogen species can be identified from the deconvolution of the N 1s peak for Ru–C₃N₄ as the sp³-hybridized tertiary N (N–(C)₃) at 399.7 eV, and the sp²-hybridized pyridinic N (C≡N–N≡C) at 398.4 eV (Figure 1f).

Again, because of ruthenium ion complexation, the binding energy of N in N–C≡N for Ru–C₃N₄ is higher than that for pristine C₃N₄ (397.8 eV, Figure S1c). The Cl 2p peak for Ru–

**Scheme 1. Schematic Illustration of Ru–C₃N₄ as a Peroxidase Mimic**

(H₂O₂)
atomic ratio of C/N in both C3N4 and RuC3N4 under the same reaction conditions. As the peroxidase-like activity of Ru−C3N4 was markedly lower than that of RuC3N4 (Figure 2b), all the catalytic experiments were conducted in the 0.10 mM acetate buffer (pH 4.5), containing 0.2 mM OPD, 1 mM H2O2, 50 µg mL−1 Ru−C3N4, and 50 µg mL−1 C3N4.

The steady-state kinetic parameters for OPD and H2O2 in the catalytic reaction were then quantitatively evaluated. From the Michaelis–Menten and Lineweaver–Burk plots in Figure S3, the Michaelis constant (Km) and maximal reaction velocity (Vmax) for Ru−C3N4 toward OPD were estimated to be 0.068 mM and 8.33 µM s−1, respectively. The small Km value indicates that Ru−C3N4 possessed a high binding affinity toward H2O2, while the large Vmax value indicates that Ru−C3N4 exhibited a high catalytic activity. Similarly, for Ru−C3N4 toward H2O2 (Figure S4), Km is estimated to be 2.4 mM and Vmax 41.66 µM s−1. The Km value toward H2O2 for Ru−C3N4 is much smaller than those for Fe3O4 nanoparticles (154 mM),12 suggesting that the Ru−C3N4 has higher binding affinity toward H2O2 than the other peroxidase mimetics reported previously. Furthermore, the Vmax value toward H2O2 for Ru−C3N4 is much larger than those for peroxidase mimetics reported previously (4.45 µM s−1 for Fe3O4 nanoparticles,31 and 3.906 µM s−1 for Pd nanoparticles,11 and 5.868 µM s−1 for Fe3O4 nanoparticles),12 signifying the high peroxidase-like activity of Ru−C3N4. In addition to the high catalytic activity, Ru−C3N4 also showed excellent stability. After storage at ambient temperature for half a year, the catalytic activity of Ru−C3N4 was found to remain virtually unchanged.

The fluorescence property of Ru−C3N4 was further examined. From Figure S5a, Ru−C3N4 can be seen to exhibit an excitation peak at 370 nm and an emission peak at 455 nm; and at 370 nm excitation, the 455 nm fluorescence, the lifetime of Ru−C3N4 increases proportionally to the concentration of Ru−C3N4 (Figure S5b). Yet, from Figure 3a, one can see that this emission is markedly quenched after incubation of 50 µg mL−1 Ru−C3N4 with 0.2 mM OPD and 1 mM H2O2 in 0.1 mM acetate buffer (pH 4.5) at 25 °C for 10 min. In addition, a new fluorescence peak emerges at 565 nm. This is due to the oxidation of OPD to fluorescent DAP catalyzed by Ru−C3N4 in the presence of H2O2. By contrast, OPD or H2O2 alone shows no influence on the fluorescence of Ru−C3N4.

To reveal the mechanism of the quenching of Ru−C3N4 fluorescence, the lifetime of Ru−C3N4 emission was measured and compared in the absence and presence of DAP. From Figure 3b, it can be seen that the fluorescence decay profiles of Ru−C3N4 remain virtually unchanged before and after the quenching by DAP, indicating no energy or electron transfer between Ru−C3N4 and DAP. Additionally, as the zeta potentials of Ru−C3N4, OPD, and DAP are all positive in 0.10 mM acetate buffer (pH 4.5) (Figure S6), it is unlikely that Förster resonance energy transfer occurred, because of electrostatic repulsion between Ru−C3N4 and DAP. From

C3N4 in Figure 1g is fitted with Cl 2p1/2 at 199.2 eV and Cl 2p3/2 at 197.6 eV, indicating the presence of chloride ions in the outer sphere to neutralize the charge. These results are consistent with those obtained previously.26 In addition, the atomic ratio of C/N in both C3N4 and Ru−C3N4 is found to be close to 3:4 by elemental analysis (Table S1).

The catalytic activity of Ru−C3N4 toward the oxidation of peroxidase substrate OPD in the presence of H2O2 was then tested in the acetate buffer (pH 4.5) at 25 °C. After incubation of the solution containing 0.2 mM OPD, 1 mM H2O2, and 50 µg mL−1 Ru−C3N4 for 10 min, the solution was found to exhibit a yellow color (Figure 2a) with a sharp absorption peak at 450 nm (Figure 2b), indicating effective catalysis of the oxidation of OPD to yellow DAP by Ru−C3N4 in the presence of H2O2 (Scheme 1). By contrast, in the absence of Ru−C3N4 or H2O2, the solution remained colorless (Figure 2a), and exhibited no apparent absorption peak at 450 nm (Figure 2b), suggesting that the direct oxidation of OPD by Ru−C3N4 or H2O2 alone was kinetically sluggish. Note that whereas C3N4 has been reported to show peroxidase-like activity,37 we found that this catalytic activity was markedly lower than that of Ru−C3N4 under the same reaction conditions. As the peroxidase-like activity of Ru−C3N4 reached the maximum at pH 4.5 (Figure S2), all the catalytic experiments were conducted in the 0.10 mM acetate buffer (pH 4.5).

Figure 1. (a, b) Representative TEM images, (c) HAADF-STEM image and elemental maps, and (d) XPS survey spectrum of Ru−C3N4. High-resolution XPS scans of the (e) Ru 3d and C 1s, (f) N 1s, and (g) Cl 2p electrons in Ru−C3N4.
C3N4-based ratiometric sensing platform was evaluated by the slope of the curve and incubation time of 10 min. OPD concentration of 0.2 mM, solution pH of 4.5, and the excitation, so the quenching of Ru
decreases accordingly, whereas the fluorescence intensity of the DAP due to the inner filter effect. Figure S8, that is, Ru–C3N4 can be concurrently quenched by the generated DAP due to the inner filter effect, Ru–C3N4 can be exploited as an effective ratiometric fluorescence probe for the detection of H2O2. To achieve a high ratiometric response toward H2O2, several important parameters were optimized (Figure S8), that is, Ru–C3N4 concentration of 50 μg mL\(^{-1}\), OPD concentration of 0.2 mM, solution pH of 4.5, and incubation time of 10 min. Under these optimized experimental conditions, the Ru–C3N4-based ratiometric sensing platform was evaluated by fluorescence measurements, where emission spectra were recorded at the excitation wavelength of 370 nm in the presence of H2O2 at different concentrations. From Figure 3c, one can see that with the increase of the H2O2 concentration, the fluorescence emission intensity of Ru–C3N4 at 455 nm decreases accordingly, whereas the fluorescence emission of DAP at 565 nm becomes intensified. Figure 3d shows the variation of the ratiometric fluorescence intensity \(\frac{I_{565}}{I_{455}}\) with H2O2 concentration, where the linear range for H2O2 detection is estimated from \(2 \times 10^{-4}\) to 1.0 mM (inset to Figure 3d), with a linear regression equation of \(y = 0.0502 + 2.97x\) \(\left(R^2 = 0.9955\right)\). The detection limit \(3\sigma/k\), with \(k\) being the slope of the curve and \(\sigma\) the standard deviation) is 50 nM, which is lower than the results in leading studies reported in recent literature (Table S2). Additionally, from Figure S9, it can be seen that at increasing glucose concentrations, the intensity of the fluorescence emission of Ru–C3N4 at 455 nm decreases accordingly, while the fluorescence emission of DAP at 565 nm increases concurrently. Figure 4b shows the ratiometric fluorescence intensity \(\frac{I_{565}}{I_{455}}\) at different glucose concentrations, which features a linear range for glucose detection from \(1 \times 10^{-3}\) to 0.5 mM (Figure 4c), with a linear regression equation of \(y = 0.0721 + 2.26x\) \(\left(R^2 = 0.9936\right)\). The detection limit is evaluated to be ca. 0.1 μM, which, again, is better than the leading literature results (Table S3).

The selectivity of the Ru–C3N4-based ratiometric sensing system was then investigated. From Figure 4d, no apparent influence was observed on the ratiometric fluorescence detection of glucose in the presence of a variety of potential interferents, such as maltose, galactose, sucrose, fructose, ascorbic acid, uric acid, lysine, glycine, and (transition) metal ions (e.g., Fe\(^{3+}\), Cu\(^{2+}\), Mg\(^{2+}\), Ca\(^{2+}\), and K\(^+\)), suggesting excellent selectivity and GOx-like catalytic specificity of Ru–C3N4. The Ru–C3N4-based ratiometric fluorescence probe was further tested for detecting glucose in human serum. As shown in Table S4, the performance of Ru–C3N4 was highly comparable to that of a commercial glucometer. The recovery efficiencies were between 95.5% and 104.3%, and the relative standard deviation was always less than 5%.
deviations were from 2.2% to 4.5%. These results suggest that the Ru–C3N4-based ratiometric sensing probe is capable of glucose detection in human serum.

## CONCLUSIONS

In conclusion, Ru–C3N4 nanosheets were found to display apparent peroxidase-like activity and behave as a peroxidase mimic to catalyze the oxidation of OPD to fluorescent DAP in the presence of H2O2. As the fluorescence emission of Ru–C3N4 was concurrently quenched by the produced DAP by virtue of the inner filter effect, an effective ratiometric fluorescence platform was constructed for the detection of H2O2 and glucose. The detection limit was estimated to be 50 nM for H2O2 and 0.1 μM for glucose. Notably, the sensing platform was successfully used for detecting glucose in human serum, highlighting the significant potential in the sensitive detection of metabolites involving H2O2-generation reactions.

## ASSOCIATED CONTENT

1. Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.9b10715.

Additional experimental data: TEM and XPS characterization of C3N4, peroxidase-like activity of Ru–C3N4 at different solution pH, kinetic investigation of Ru–C3N4 toward OPD, kinetic investigation of Ru–C3N4 toward H2O2, fluorescence spectra of Ru–C3N4, zeta potentials of Ru–C3N4, OPD, and DAP, UV–vis absorption spectrum of DAP and fluorescence emission spectrum of Ru–C3N4, optimization of experimental parameters for H2O2 detection, selectivity of H2O2 detection, optimization of experimental parameters for glucose detection, elemental analysis results, performance comparison of H2O2 detection, performance comparison of glucose detection, and comparison of glucose detection in human serum with a commercial glucometer (PDF)

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W.D. and Y.P. contributed equally to the work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

Work at HNNU was supported by the National Natural Science Foundation of China (21705045 and 21775041), the Science and Technology Innovation Project of Hunan Province (2018RS3062), the Natural Science Foundation of Hunan Province (2018JJ2252), the Scientific Research Fund of Hunan Provincial Education Combination Department (17A125), and the Science and Technology Project of Changsha (KQ1802036). Work at UCSC was supported by the National Science Foundation through grants CHE-1710408 and CBET-1848841. W.F.D. and Y.M.T. were supported by a fellowship from the China Scholarship Council.

### REFERENCES


