Turn-on Luminescent Probe for Hydrogen Peroxide Sensing and Imaging in Living Cells based on an Iridium(III) Complex–Silver Nanoparticle Platform

Jinshui Liu¹,², Zhen-Zhen Dong², Chao Yang³, Guodong Li³, Chun Wu², Fu-Wa Lee⁴, Chung-Hang Leung³ & Dik-Lung Ma²

A sensitive turn-on luminescent sensor for H₂O₂ based on the silver nanoparticle (AgNP)-mediated quenching of an luminescent Ir(III) complex (Ir-1) has been designed. In the absence of H₂O₂, the luminescence intensity of Ir-1 can be quenched by AgNPs via non-radiative energy transfer. However, H₂O₂ can oxidize AgNPs to soluble Ag⁺ cations, which restores the luminescence of Ir-1. The sensing platform displayed a sensitive response to H₂O₂ in the range of 0−17 μM, with a detection limit of 0.3 μM. Importantly, the probe was successfully applied to monitor intracellular H₂O₂ in living cells, and it also showed high selectivity for H₂O₂ over other interfering substances.

H₂O₂ is widely used in industry and households for rinsing, bleaching and disinfection. For example, in the food industry, H₂O₂ is used to replace chlorine-containing bleaching and sterilizing agents⁵. It also plays an important role in many biological processes and enzymatic reactions, particularly those related to intracellular oxidative stress⁶. In fact, escalated levels of H₂O₂ can cause irreversible cellular damage through the oxidation of biomolecules, leading to cell death⁷. Moreover, oxidative damage to cellular proteins, nucleic acids, and lipid molecules are associated with aging and age-related disorder ranging from neurodegeneration to diabetes⁸,⁹. Therefore, a rapid and reliable detection of H₂O₂ is important in pharmaceutical, clinical, and food industries.

Multiple methods such as spectrophotometry⁵,⁶, chemiluminescence⁷ and electrocatalysis⁸ have been developed for the detection of H₂O₂. Specifically, biosensors have been developed on the basis of electrocatalysis of immobilized enzymes arising from H₂O₂ reduction⁹. However, the enzyme-based biosensors are limited by sensitivity to environmental conditions, high cost, short shelf-life and complicated immobilization procedures⁸,¹⁰–¹². Meanwhile, fluorescent strategies have lots of advantages, particularly rapid response, high sensitivity, and simple manipulation¹³,¹⁴. Various fluorescence probes such as organic molecules¹⁵, carbon dots¹⁶,¹⁷, metal nanoclusters¹⁸, and nanoparticles¹⁹–²¹, have good performance on the determination of H₂O₂. However, there are still some drawbacks for these reported probes, including poor sensitivity and selectivity, low stability in biological environment, or complicated operation¹⁷,¹⁸,²². Fluorescence turn-on sensors are generally more desirable than fluorescence quenching sensors as the former is less susceptible to false positive signals²³,²⁴.

Luminescent Ir(III) complexes have been employed to detect a variety of analytes²⁵–²⁷. Compared with organic molecules, Ir(III) complexes generally exhibit large Stokes shifts, ease in synthesis and long-lived luminescence which could be distinguished from fluorescence noise in biological matrices²⁶,²⁷. Meanwhile, silver nanoparticles (AgNPs) form a promising nanomaterial that has been developed in many applications because of their remarkable properties, such as high extinction coefficient and surface plasmon resonance absorption²⁸–³⁰. It has been reported that AgNPs can be oxidized by traces of H₂O₂, to form Ag⁺³¹. In addition, AgNPs can function as

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excellent quenchers for fluorescent materials, such as organic dyes and quantum dots (QDs)\textsuperscript{32–35}. However, as far as we know, the application of the Ir(III) complexes combined with AgNPs has not yet been reported in the literature for H\textsubscript{2}O\textsubscript{2} sensing. Consequently, taking advantages of the Ir(III) complex (Ir-1, [Ir(tfppy)\textsubscript{2}(pyphen)]\textsuperscript{+}, where tfppy = 2-[4-(trifluoromethyl)phenyl]pyridine, pyphen = pyrazino[2,3-f][1,10]phenanthroline) and AgNPs, we designed a novel turn-on luminescent probe for rapid and sensitive detection of intracellular H\textsubscript{2}O\textsubscript{2}.

### Results and Discussion

#### Sensing Mechanism

Ir-1, carrying tfppy as its C\textsuperscript{N} ligand and pyphen as its N\textsuperscript{N} ligand (Fig. 2a), was characterized by\textsuperscript{\textsuperscript{\textsuperscript{\textsuperscript{\textsuperscript{1\textsuperscript{H}-NMR,\textsuperscript{13}C-NMR and HRMS (Figs S1–S3 and Table S1).} }}\textsuperscript{Ir-1}}\textsuperscript{emits strong luminescence

The sensing mechanism of the Ir-1–AgNP probe for H\textsubscript{2}O\textsubscript{2} is illustrated in Fig. 1. In the initial system, the luminescence of Ir-1 was significantly quenched by AgNPs. However, this AgNPs-induced quenching effect can be reversed by H\textsubscript{2}O\textsubscript{2} due to oxidation of AgNPs to Ag\textsuperscript{+}. To our knowledge, the Ir-1–AgNP is the first application of the combination of Ir(III) complexes and AgNPs for H\textsubscript{2}O\textsubscript{2} sensing in both aqueous solutions and living cells.

Figure 1. Illustration of the design rationale for the detection of H\textsubscript{2}O\textsubscript{2} using a luminescence sensor based on Ir-1–AgNPs system.

Figure 2. (a) Chemical structure of Ir-1. (b) Luminescence emission spectra 0.3 \textmu M Ir-1 in Tris-HNO\textsubscript{3} buffer solution (pH 7.0) containing different concentrations of AgNPs. The inset is the luminescence intensity at 545 nm plotted against the AgNPs concentration.

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Fig. S4, the luminescence intensity of the Ir-1–AgNP system increased with time and reached the plateau after 10 min, indicating that the reaction between AgNPs and H$_2$O$_2$ at ambient temperature is rapid. In the absence of AgNPs, H$_2$O$_2$ showed no apparent effect on the luminescence of Ir-1 (Fig. S5). Therefore, the increase in the luminescence of the system should arise primarily from the decomposition of AgNPs by H$_2$O$_2$, which restores the emission of Ir-1.

The mechanism involved in the luminescence quenching and recovery process was also demonstrated by transmission electron microscopy (TEM) imaging. In the absence of the Ir-1, the AgNPs were well-dispersed (Fig. 3a). However, after the addition of Ir-1, slight aggregation of AgNPs was observed, suggesting that Ir-1 and AgNPs interacted on the surface of AgNPs (Fig. S6b). The identity of the Ir-1–AgNP complex was further confirmed by energy dispersive X-ray spectroscopy (EDX), which showed strong elemental signals for both Ir and Ag (Fig. S6c). Strikingly, after treatment of AgNPs with H$_2$O$_2$, no AgNPs could be observed in the TEM images (Fig. 3b). This suggests that the AgNPs were decomposed and transformed to Ag$^+$, which is consistent with previously reported studies. The UV–vis absorbance spectra of AgNPs in the absence and presence of H$_2$O$_2$ are shown in Fig. S7. AgNPs alone showed a strong characteristic surface plasmon resonance peak at around 390 nm. However, the absorption band of AgNPs gradually decreased upon increasing concentration of H$_2$O$_2$. These phenomena were ascribed to the oxidation of AgNPs to Ag$^+$ by H$_2$O$_2$, leading the decomposition of the AgNPs.

**Sensitivity.** To explore the applicability of the proposed luminescence sensor for H$_2$O$_2$ detection, we studied the luminescence response of the Ir-1–AgNP system toward varying concentrations of H$_2$O$_2$. The luminescence intensity of the system was gradually restored with increasing concentration of H$_2$O$_2$ (Fig. 4a). Meanwhile, a good linear relationship over the range from 0 to 17 μmol L$^{-1}$ with a correlation coefficient of 0.998 was obtained (Fig. S8). The limit of detection (LOD) was calculated to 0.3 μM according to the signal-to-noise method.
(S/N = 3). The sensitivity of this method is comparable to other reported methods for H$_2$O$_2$ detection as summarized in Table S24, 10–16, 20, 36, 40–44.

Selectivity. To assess the selectivity of Ir-1–AgNPs system for H$_2$O$_2$, the influences of metal ions and amino acids were studied. As shown in Fig. 4b, nearly no luminescence changes could be observed with the other substances (Fig. 4b), which demonstrates that the Ir-1–AgNP system is highly selective for H$_2$O$_2$ over other non-target substances.

Cell imaging. Given the promising capability of Ir-1 for sensing H$_2$O$_2$ in aqueous solution, we then investigated the ability of Ir-1 for monitoring H$_2$O$_2$ in living human cells. Ir-1 showed cytotoxicity against HeLa (human cervical cancer) cells with an IC$_{50}$ value of 5.12 μM (Fig. S9).

In the cell imaging study, the luminescence intensity of HeLa cells was enhanced with increasing concentration of Ir-1 (Fig. 5a), showing that Ir-1 could effectively penetrate into cells. A concentration of 0.3 μM of Ir-1 was chosen for subsequent cell experiments as this concentration was over 10-fold lower than the IC$_{50}$ value for cytotoxicity, while it still gave a good luminescence signal.

Next, HeLa cells were pretreated with Ir-1 (0.3 μM) for 1 h before incubation with different concentration of AgNPs. The luminescence intensity of HeLa cells was remarkably reduced with increasing concentration of AgNPs (Fig. S10), which was attributed to AgNPs-mediated quenching of an luminescent Ir-1 as described previously. However, when H$_2$O$_2$ was added into the growth medium for another 1 h, the luminescence of HeLa cells was recovered in a dose-dependent manner (Fig. 5b). Collectively, these results suggest that Ir-1–AgNP can be developed for the monitoring of H$_2$O$_2$ levels in living cells.

Figure 5. Confocal luminescence microscopy imaging of HeLa cells. (a) HeLa cells were incubated with the indicated concentration of Ir-1 for 1 h. (b) HeLa cells were pretreated with Ir-1 (0.3 μM) and AgNPs (2.8 μM) for 1 h before incubation with different concentration of H$_2$O$_2$. The upper row is luminescence imaging, and the lower row is bright field imaging. Excitation wavelength = 405 nm.
**Conclusion**
Consequently, we have proposed a turn-on luminescence assay for \( \text{H}_2\text{O}_2 \) detection employing the \( \text{Ir-1–AgNP} \) system. In this nano-composite system, \( \text{Ir-1} \) functioned as a luminescence reporter, while AgNPs were employed both as a luminescence quencher and as a recognition unit for \( \text{H}_2\text{O}_2 \). Based on the luminescence recovery of the \( \text{Ir-1–AgNP} \) system triggered by \( \text{H}_2\text{O}_2 \), this nanoprobe was successfully applied to detect \( \text{H}_2\text{O}_2 \) at the intracellular level in living cells. In addition, the \( \text{Ir-1–AgNP} \) probe possesses some superior properties, including label-free, good sensitivity and selectivity, low cost, easy manipulation, low cytotoxicity, and turn-on luminescent response. To our knowledge, the probe is the first combination of \( \text{Ir(III)} \) and AgNPs applied for the detection of \( \text{H}_2\text{O}_2 \) in living cells reported in the literature.

**Materials and Methods**

**Chemicals and materials.** Iridium chloride hydrate (\( \text{IrCl}_3\cdot\text{xH}_2\text{O} \)) was purchased from Precious Metals Online (Australia). Other reagents were purchased from Sigma Aldrich (St. Louis, MO) and used as received. All of the reagents were of analytical grade and were used as received without further purification. All solutions were prepared in Milli-Q water under ambient conditions. HeLa cell lines were obtained from ATCC (Manassas, VA, USA). Dulbecco’s Modified Eagle’s medium, fetal bovine serum, penicillin and streptomycin were obtained from Sigma-Aldrich Co. LLC (St. Louis, MO, USA).

**Synthesis of AgNPs.** AgNPs were fabricated according to reported methods with slight modifications.\(^{28,45}\) In a typical procedure, 0.08 mL \( \text{AgNO}_3 \) (0.1 M) and 0.1 mL trisodium citrate (0.1 M) were mixed into 100 mL pure water and stirred under the condition of ice bath. Then, freshly prepared \( \text{NaBH}_4 \) solution was added drop-wise into the mixture until it turned yellow. The resulting yellow solution was stirred for another 30 min to form AgNPs quantitatively, which was stored at 4°C for subsequent use. The diameter of AgNPs prepared was measured to be 8–9 nm by transmission electron microscopy (TEM).

**Synthesis of \( \text{Ir-1} \).** \( \text{Ir-1} \) was synthesized based on a reported literature method\(^{46–49}\). \( \text{[Ir}(\text{tpfppy})\text{Cl}_2] \) (0.2 mmol) and ppy (0.42 mmol) in a mixed solvent of DCM:methanol (1:1.2 (v/v), 36 mL) was refluxed overnight. The reaction mixture was allowed to cool to ambient temperature, and unreacted cyclometallated dimer was removed by filtration. Excess ammonium hexafluorophosphate was then added into the filtrate, and the resulting mixture was stirred for another 30 min. Afterwards, the solution was evaporated under reduced pressure until precipitation was initiated. The precipitate was filtered, and washed by several portions of water and diethyl ether. The crude product was then recrystallized by the acetonitrile/diethyl ether vapor diffusion to obtain the desired compound, which was characterized by \( \text{H}-\text{NMR}, \text{13C}-\text{NMR}, \text{HRMS} \) and elemental analysis.

**Luminescence response of \( \text{Ir-1} \) towards AgNPs.** \( \text{Ir-1} \) (0.3 \( \mu \text{M} \)) was added to varying concentrations of AgNPs in Tris-\( \text{HNO}_3 \) buffer (5 mM Tris-\( \text{HNO}_3 \), pH 7.0), then their emission intensity were measured.

**Detection of \( \text{H}_2\text{O}_2 \).** A series of sample solutions of same composition was prepared by mixing \( \text{Ir-1} \) (0.3 \( \mu \text{M} \)) with AgNPs (2.8 \( \mu \text{M} \)) in Tris-\( \text{HNO}_3 \) buffer (5 mM Tris-\( \text{HNO}_3 \), pH 7.0). Upon individual addition of varying concentrations of stock \( \text{H}_2\text{O}_2 \) solution, the sample solutions were incubated for 10 min at room temperature. Emission spectra were collected in the range of 450–700 nm at the excitation wavelength of 295 nm.

**Cell imaging.** HeLa cells were pretreated with \( \text{Ir-1} \) (0.3 \( \mu \text{M} \)) for 1 h at 37 °C, then AgNPs of different concentrations (0 \( \mu \text{M}, 0.1 \mu \text{M}, 0.3 \mu \text{M}, 1 \mu \text{M}, 3 \mu \text{M} \) and 5 \( \mu \text{M} \)) was added before further incubation for 1 h. After washing with PBS three times, the luminescence intensity of HeLa cells was imaged by a Leica SP8 laser scanning confocal microscope upon excitation at 405 nm. For \( \text{H}_2\text{O}_2 \) detection, the experiment was performed as above except that after incubation in the presence of AgNPs (2.8 \( \mu \text{M} \)), cells were further treated with \( \text{H}_2\text{O}_2 \) ranging from 0 to 20 \( \mu \text{M} \) for 1 h. After washing with PBS three times, the luminescence intensity of HeLa cells was then imaged as above.

**Statistics analysis.** One-way analysis of variance (ANOVA) followed by the Dunnett’s method for multiple comparisons by using GraphPad Prism 6.0 was used to analyse the data.

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Author Contributions

Jinshui Liu contributed to the sensing experiments and manuscript draft; Zhen-Zhen Dong prepared the iridium complex, SEM and TEM experiments; Chao Yang and Guodong Li completed the cell imaging and MTT experiments; Chun Wu helped to draw and arrange the Figures 1–5 in the manuscript; Fu-Wa Lee mainly commented and revised the manuscript; Chung-Hang Leung and Dik-Lung Ma proposed and instructed the project.

Additional Information

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