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COMMUNICATION

A long-lifetime iridium(III) complex for lysosome tracking with high specificity and a large Stokes shift

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Abstract

Investigating the role of lysosome dysfunction in cancer requires the development of efficient probes for lysosomes. We report herein a cyclometalated iridium(III) complex (Ir-Ly) as a luminescent probe for visualizing lysosomes in cancer cells. The morpholine and hydroxy moieties within Ir-Ly provide suitable hydrophilicity and responsiveness to pH. Importantly, Ir-Ly exhibits large Stokes shift, long lifetime and high photostability, which are important advantages for lysosome tracking in living cells.

Introduction

Lysosomes are essential organelles that are responsible for cellular digestion. They break down extracellular materials taken up by the cell as well as intracellular components under specific circumstances in order to regulate the cellular dynamic physiological balance.[1, 2] There are over 50 hydrolases in lysosomes which are responsible for carrying out the degradation activities of lysosomes. As lysosomes and lysosomal enzymes play important functions in programmed cell death, considerable attention has been drawn towards the role of lysosomes in cancer.[3, 4] These studies demand the development of new kinds of molecular probes for examining lysosomal function and dysfunction within the cell.[5]

Fluorescence microscopy is a widely used technology used for non-invasive imaging of organelles in living cells. The ability to track and image lysosomes accurately could be useful for the monitoring of lysosome behavior in cancer cells.[6] However, most commercially accessible probes for lysosomes are based on fluorescent organic dyes, which suffer from poor photostability,[7, 8] limiting the time period for observing dynamic changes of lysosome behavior. Furthermore, other long-term fluorescent probes for lysosomes reported in the literature, including dextran-linked fluorophores,[9] inorganic/hybrid nanoparticles,[10] and organic dyes,[11, 12] required excitation with short wavelengths leading to unwanted autofluorescence, photobleaching, photodamage, and shallow depth of penetration. Inorganic luminescent materials have emerged as promising alternatives to fluorescent dyes for cellular imaging due to their low phototoxicity, good photostability, deeper penetration, large Stokes shifts and long luminescent lifetimes.[13-15]

Based on our continuing interest in the development of luminescent iridium(III) complex probes,[16-18] we report in this work our efforts to develop a iridium(III) complex for lysosome tracking. We designed and synthesized an iridium(III) complex **Ir-Ly**, which incorporates morpholine and hydroxy groups in the phenanthroline N^N ligand, and also bears two phenylpyridine C^N co-ligands. The morpholine moiety is crucial for lysosomal localization,[19] as the protonation of **Ir-Ly** within the acidic interior of the lysosome converts the complex into a more hydrophilic species that is retained within the lysosome. Therefore, the protonated morpholine moiety acts as a "lock" that allows **Ir-Ly** to accumulate in lysosomes, allowing the probe to be used for observing lysosomes in cancer cells (Fig. 1a). Meanwhile, the hydroxy groups in the complex can act as a sensing unit in basic solution, and also confer increased water solubility (Fig. 1b).

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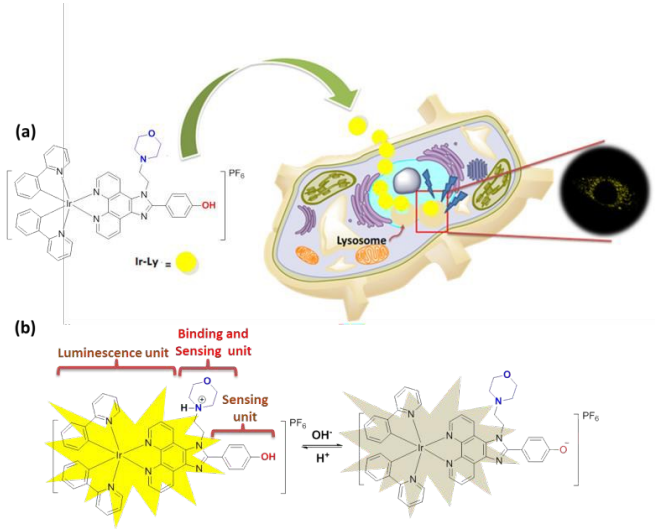

Fig. 1 **Ir-Ly** for lysosome tracking. (a) **Ir-Ly** enters and is retained in lysosomes. (b) Schematic of H^+ and OH^- sensing by **Ir-Ly**.

Results and discussion

Synthesis of Ir-Ly

Complex **Ir-Ly** was prepared through four steps as depicted in Fig. 2. 1,10-Phenanthroline-5,6-dione **1** was reacted with 4-methoxybenzaldehyde **2** in a simple cyclo-condensation reaction to produce compound **3**. Then, alkylation of **3** produced the morpholine derivative **4**, followed by demethylation to reveal the hydroxy groups in the phenanthroline N^N ligand **5**. Finally, the reaction of N^N ligand **5** with the organometallated dimer $[Ir(ppy)_2Cl]_2$ and subsequent anion exchange with NH_4PF_6 generated the iridium(III) complex **Ir-Ly** in 65% yield. The intermediates **3-5** and complex **Ir-Ly** were characterized by ^1H NMR, ^{13}C NMR, and HRMS spectrometry (see SI).

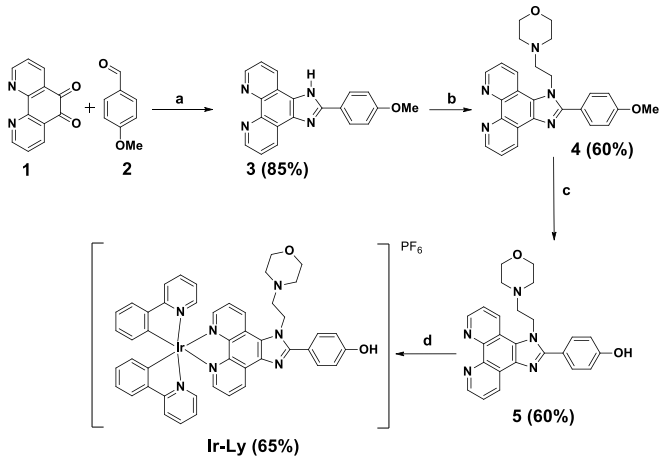

Fig. 2. Synthesis of complex **Ir-Ly**. Reagents and conditions: a) NH_4OAc/AcOH, reflux, overnight, 85%; b) 4-(2-chloroethyl)morpholine, NaH/DMF, r.t. to reflux, 25 h, 60%; c) 2-aminobenzenethiol, Cs_2CO_3, NMP, 185 °C, 3 h, 60%; d) $[Ir(ppy)_2Cl]_2$, DCM/MeOH (1:1), r.t., overnight, 65%.

Photophysical properties of Ir-Ly

With the complex in hand, the optical characteristics of **Ir-Ly** were next investigated. **Ir-Ly** showed excitation and emission maxima of 290 and 590 nm, respectively (Fig. S3†). Its large Stokes shift of 300 nm is larger than the Stokes shift of typical organic dyes, which reduces the likelihood of self-quenching. Additionally, **Ir-Ly**'s luminescence lifetime was relatively long-lived (*ca.* 4.2 µs) (Table S1), which is substantially longer than lifetimes of organic dyes (typically ns). Therefore, the emission of complex **Ir-Ly** could easily be distinguished from background autofluorescence through the use of time-resolved emission spectroscopy (TRES). To verify this hypothesis, the luminescent properties of **Ir-Ly** were monitored in the presence of coumarin-460 (CM460), as a typical fluorescent dye. In steady-state mode, the luminescence of **Ir-Ly** was disturbed by the robust emission of CM460 at about 460 nm, reducing the precision of determination (Fig. 3a). However, when the delay was set to 333 ns, which is long after the completion of the fluorescence decay of CM460 (3.4 ns),[20] the emission from CM460 was almost entirely absent and only the emission of complex **Ir-Ly** could be detected (Fig. 3b). These results establish the possible application of **Ir-Ly** in naturally fluorescent milieus, via the use of TRES, thanks to its long-lived luminescence.

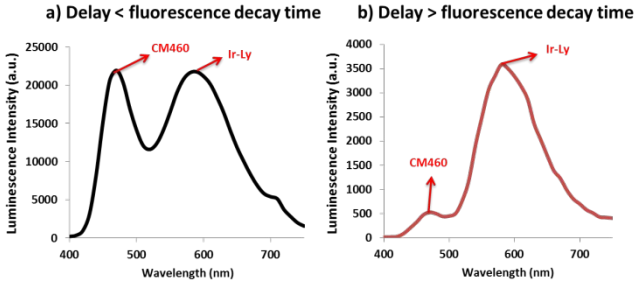

Fig. 3. TRES of complex **Ir-Ly** (20 µM) in the presence of CM460 (2 µM) in PBS buffer (0.1 mM, pH 4.5) under an excitation wavelength at 355 nm; time gate (a) Delay < 333 ns or (b) Delay > 333 ns.

pH effect and specificity study

The luminescent intensity of **Ir-Ly** was responsiveness to pH. As pH increased, the emission intensity of **Ir-Ly** was reduced (Fig. 4). This is due to the ability of morpholine, as an electron donor, to quenching the luminescence of the iridium(III) center through photoinduced electron transfer (PET). However, since morpholine has a pK_a of 5−6,[21] it will become protonated in acidic conditions. This prevents PET, leading to an enhancement of luminescence emission at low pH.

As specificity is an important characteristic of a probe, we investigated the selectivity of **Ir-Ly** by introducing different concentrations of sodium ions, various cations, anions or amino acids into a solution of **Ir-Ly** (Fig. S1). Encouragingly, the results revealed that **Ir-Ly** had no obvious response towards any of the tested interferents. Such emission response of pH change and specificity are important for indicating the location and morphology of lysosomes.

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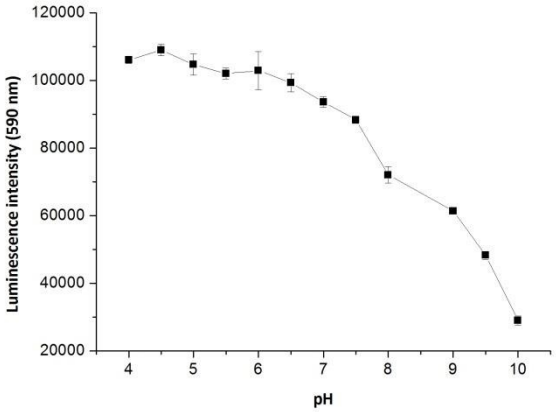

Fig. 4. Emission intensity of **Ir-Ly** at 590 nm at different pH values in PBS buffer.

Visualization of lysosomes in living cells

Lysosomes are more acidic organelles (pH 5.0-5.5) compared with cytoplasmic or other subcellular compartments (pH *ca.* 7.4).[22] In consideration of responsiveness of **Ir-Ly** to pH, as well as the promising luminescent behavior of the complex in aqueous media, we hypothesized that **Ir-Ly** could function as an ideal probe for lysosomes. To study this, the ability of the probe to track lysosomes in living cells was investigated. First, the cytotoxicity of complex **Ir-Ly** towards human cervical cancer (HeLa) cells was first investigated using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Complex **Ir-Ly** showed negligible cytotoxicity (IC_{50} value > 200 μM) after 6 h (Fig. S2), indicating that it could be used for tracking lysosomes in living cells without adverse effects on their normal function under the typical time and concentration parameters employed.

We next explored the ability of **Ir-Ly** for lysosome tracking in living HeLa cells. Cells were stained with the indicated concentrations of **Ir-Ly** for 1 h in a CO_2 incubator at 37 °C, followed by a subsequent incubation with the lysosome-staining Lyso-Tracker dye for 30 min and the nuclear-staining dye Hoechst 33342 for another 10 min. Cells were washed three times with phosphate buffer, then luminescence images were recorded using a confocal laser scanning microscope with excitation at 405 nm. **Ir-Ly** exhibited a yellow punctuated luminescence that was enhanced with increasing complex concentration (Fig. 5). The co-localization of **Ir-Ly** with Lyso-Tracker suggested that **Ir-Ly** could selectively monitor lysosomes in living HeLa cells.

The ability of **Ir-Ly** to monitor lysosomes in different kinds of living cells was further validated using a human non-small-cell lung cancer cell line (A549) and a normal human umbilical vein endothelial cell line (HUVEC) (Fig. 6). Notably, **Ir-Ly** appeared to show even higher specificity for lysosomes in both cell lines, as evidenced by the accumulation of the yellow punctuated luminescence in the cytoplasm, compared to Lyso-Tracker, which exhibited slight fluorescence in the nuclear region where lysosomes are not expected to be found. Taken together, these results indicate that the luminescent probe **Ir-Ly** could selectively monitor lysosomes in both carcinoma and normal living human cell lines.

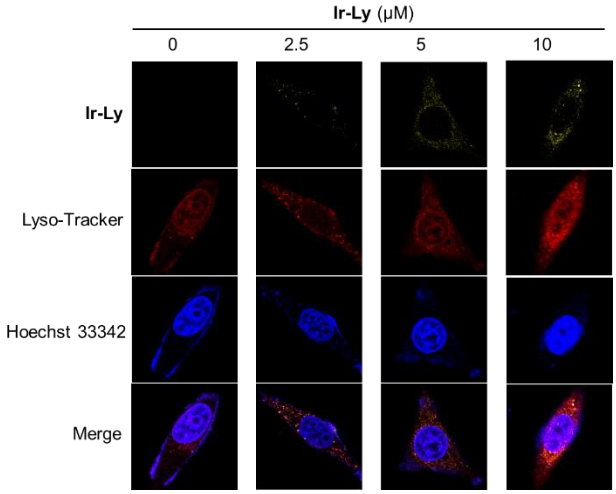

Fig. 5. Luminescence images of HeLa cells incubated with different concentrations of **Ir-Ly** (0, 2.5, 5, and 10 μM). After incubation with **Ir-Ly** for 1 h at 37 °C, cells were washed three times with phosphate buffer and stained with Lyso-Tracker Red (1:15,000) for 30 min and Hoechst 33342 (1:2,000) for 10 min. Luminescence images were captured under an excitation wavelength of 405 nm.

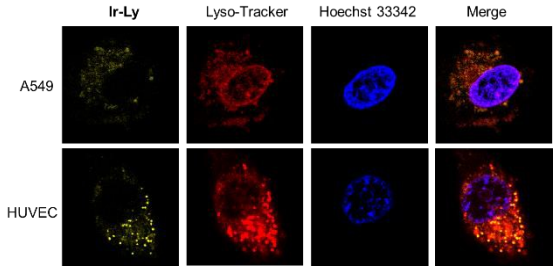

Fig. 6. Intracellular co-localization of **Ir-Ly** (10 μM) and Lyso-Tracker (1:15,000) in cancer (A549) and normal (HUVEC) living cells. Luminescence images of cells were captured under an excitation wavelength of 405 nm.

Conclusions

In conclusion, we have designed and synthesized a cyclometalated iridium complex **Ir-Ly** as an effective luminescence lysosomal tracker. As a consequence to its large Stokes shift, weak cytotoxicity, and specific localization in lysosomes, **Ir-Ly** can be used to track lysosomes in both carcinoma and normal living cell lines. Further studies for imaging of other organelles and biomedical applications with the long-lived luminescent iridium(III) complexes are ongoing in our laboratories.

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Conflicts of interest

In accordance with our policy on Conflicts of interest please ensure that a conflicts of interest statement is included in your manuscript here. Please note that this statement is required for all submitted manuscripts. If no conflicts exist, please state that "There are no conflicts to declare".

Notes and references

‡ Footnotes relating to the main text should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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