Development of luminescent iridium(III) complex-based probes for monitoring analytes in environmental and biological systems

Wanhe Wang

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Issued by Graduate School, HKBU
Development of Luminescent Iridium(III) Complex-based Probes for Monitoring Analytes in Environmental and Biological Systems

WANG Wanhe

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Principal Supervisor:
Dr. MA Edmond Dik Lung (Hong Kong Baptist University)

July 2019
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of PhD at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institutions for a degree, diploma or other qualifications.

I have read the University’s current research ethics guidelines, and accept responsibility for the conduct of the procedures in accordance with the University’s Research Ethics Committee (REC). I have attempted to identify all the risks related to this research that may arise in conducting this research, obtained the relevant ethical and/or safety approval (where applicable), and acknowledged my obligations and the rights of the participants.

Signature:  

Date: July 2019
ABSTRACT

Transition metal complexes offer potential alternatives to fluorescent organic compounds in various sensing applications. They show several characteristic properties over organic dyes, such as strong luminescence emission, long emission lifetime and large Stoke shift. Among transition metal complexes, cyclometalated iridium(III) (Ir) complexes are most widely explored for sensing applications, due to their bright and tuneable phosphorescence emission. Up to now, Ir(III) complexes have been successfully applied to detect a range of analytes in environmental and biological systems, such as cations, anion, small molecules and proteins. In this thesis, we deeply explored the capability of Ir(III) complexes to detecting a range of targets including metal ions, small molecules and biomarkers. Several strategies are used to improve the biocompatibility of Ir(III)-based probes while retaining their desirable characteristics.

In chapter 2, we developed a novel Ir(III) complex for the detection of Al$^{3+}$ with a detection limit of 1 μM. The long lifetime of the complex was harnessed to distinguish luminescence response to Al$^{3+}$ from autofluorescence in biological samples by TRES experiment, while the probe was also successfully applied for imaging Al$^{3+}$ in living cells. The results have been published as Chem. Commun., 2016, 52, 3611. In chapter 3, we reported a new reaction-based luminogenic probe for imaging both H$_2$S and hypoxia in living zebrafish. This probe demonstrated their utility for the detection of H$_2$S in solution, living cells and zebrafish model, while it was also capable of discriminating hypoxic from normoxic cells and zebrafish model. The results have been published as Sens. Actuator B-Chem., 2018, 255, 1953. In chapter 4, we conjugated a natural product oridonin to an Ir(III) scaffold for tracking intracellular NF-κB. This complex was successfully applied to track NF-κB translocation induced by TNF-α, without affecting the translocation process. The results have been published as Chem. Eur. J., 2017, 23, 4929. In chapter 5, an Ir(III) scaffold with galactose moiety was designed and synthesized for discriminating ovarian carcinoma cell lines from normal cell lines. This probe can selectively “light up” ovarian carcinoma cells with negligible luminescence in normal cells. The results have been published as Anal. Chem., 2017, 89, 11679.

These works have further demonstrated the utility of Ir(III) complex in the monitoring environment and studying biomolecules in living systems. In particular, the conjugation of endogenous molecule galactose or a natural compound oridonin to Ir(III) scaffolds highlights an effective solution to develop biocompatible probes. However, it should be pointed out that there is a need for developing a general strategy to improve the biocompatibility of luminescent Ir(III) complex-based probes, while there is huge potential for incorporating luminescent Ir(III) complexes-based sensing platforms into portable devices, and exploring theranostic probes.
ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my principal supervisors, Dr. Dik-Lung MA (Hong Kong Baptist University) and Prof. Chung-Hang LEUNG (University of Macau), for their concerns and encouragement that support me, not only in the research study but also in my personal life, over past three years. I would like to express my deepest thanks to my co-supervisor, Dr. Kangning REN (Hong Kong Baptist University), for his helpful guidance during my Ph.D. study. Their guidance helped me in all the time of research and preparation of my thesis. In addition, I would like to thank the technicians in Department of Chemistry and my group members (Dr. Suk-Yu WONG, Dr. Daniel Shiu-Hin CHAN, Dr. Kasipandi Vellaisamy, Dr. Ka-Ho LEUNG, Dr. Lihua LU, Dr. Modi WANG, Dr. Chao YANG, Dr. Tianshu KANG, Chung Nga KO, Guodong LI, Chun WU) for their kind help in my experiments. Lastly, my special thanks would be given to my families and Jing for supporting me throughout these years. Without their support, this accomplishment may not be able to come true.
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Figure 4.1 Synthesis of complex 1. Reagents and conditions: a) ammonium acetate, HOAc, reflux, overnight, 75%; b) LiOH•H₂O, MeOH, H₂O, reflux, 5 h, 90%; c) SOCl₂, reflux, 3 h; (d) oridonin, Et₃N, DCM, rt, overnight, 60%; e) Ir(III) dimer [Ir(ppy)₂Cl]₂, DCM/MeOH (1/1), rt, overnight, then NH₄PF₆, 64%.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>BODIPY</td>
<td>Boron-dipyrromethene</td>
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<tr>
<td>CO</td>
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<td>Ni</td>
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<td>MLCT</td>
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<td>HOMO</td>
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<td>Cesium ion</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>Li⁺</td>
<td>Lithium ion</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>Manganese ion</td>
</tr>
<tr>
<td>Cr³⁺</td>
<td>Chromic ion</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>Barium ion</td>
</tr>
<tr>
<td>RSS</td>
<td>Reactive sulfur species</td>
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<tr>
<td>H₂S</td>
<td>Hydrogen sulfide</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
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<td>Cys</td>
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<td>Glutathione disulfide</td>
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<td>CSSC</td>
<td>Cystine</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>CBS</td>
<td>Cystathionine beta-synthase</td>
</tr>
<tr>
<td>HRMS</td>
<td>High-resolution mass spectrometry</td>
</tr>
<tr>
<td>NTR</td>
<td>Nitroreductase</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>Cobalt(II) chloride</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-tetrazolium bromide</td>
</tr>
<tr>
<td>ELISA</td>
<td>(enzyme-linked immunosorbent assay) is a plate-based assay</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase complex</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>hpf</td>
<td>Hour post-fertilization</td>
</tr>
<tr>
<td>XTT</td>
<td>2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-Galactosidase</td>
</tr>
<tr>
<td>ThT</td>
<td>Thioflavin T</td>
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<tr>
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<td>Hydrochloric acid</td>
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<tr>
<td>Cs₂CO₃</td>
<td>Caesium carbonate</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
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</table>
Chapter 1

Introduction

1.1 Brief introduction of fluorescence probes

Fluorescence is a process describing the excitation of a fluorophore to its excited state through photons absorption, and the relaxation to its ground state releasing photons.\(^1\) Fluorescence was first observed by an Anglo-Irish physicist George Stokes in 1852, and then it was physically described by a Polish physicist Alexander Jablonski in 1935.\(^2,3\) Nowadays, fluorescence technique has become an indispensable analytical tool in environmental monitoring and biological studies, due to its low cost, high sensitivity, and other valuable advantages. For example, fluorescence enables single-molecule tracking biomolecules in living cells.\(^4\) This technique also can track a biological process in a biomolecular level with high spatial and temporal resolution. Fluorescence technique is also compatible with other techniques including microfluidic chip, allowing \textit{in situ} analysis. Due to poor inherent fluorescence of analytes, fluorescence technique generally needs fluorophores to provide a simple and straight ways to detect targets, identify targets and monitor molecular interactions, so current fluorescence techniques mainly rely on fluorescent proteins, small-molecule dyes and luminescent nanoparticles.\(^2\) In particular, the discovery of fluorescent proteins (e.g. GFP) initiated a renaissance of fluorescence microscopy, allowing to genetically introducing a fluorescent protein tag to a protein of interest in living systems.\(^5,6\)
However, fluorescent proteins also suffer from inevitable disadvantages, such as large molecular size and potential perturbation to the biological system. Therefore, small-molecule dyes are of particular importance due to their relatively small molecular size, ease of modification on chemical structures and tuneable properties.\(^7\) Up to now, various small-molecule scaffolds have been reported to study analytes in solution and living systems, such as BODIPY, fluorescein, cyanine, and coumarin.\(^7\) However, the disadvantages of these small-molecule scaffolds including photobleaching initiate the exploit for new alternatives.

### 1.2 Brief introduction of luminescent transition metal complexes

Organotransition metal complexes are compounds featuring in bonds between carbon and a transition metal.\(^8\) Transition metals consist of groups 3–12 with a partially filled \(d\) sub-shell, allowing them to coordinate with ligands to form coordination compounds. These characteristic properties of transition metals enable them to be utilized in different applications such as catalysis, functional materials, and probes. In early 1880s, transition metal organometallic chemistry was first applied in industry, when Ludwig Mond utilized the formation of gaseous Ni(CO)\(_4\) between CO nickel to purify nickel, due to the ease of separation of Ni(CO)\(_4\) from solid impurities, which was then subjected to be thermally decomposed to produce pure nickel.\(^9\)

Nowadays, bioorganometallic chemistry has become a sub-discipline of organometallic chemistry, serving as an important supplement for other
well-known organometallic areas such as catalysis and materials. Much effort has been devoted to bioorganometallic field, identifying various topics, including organometallic compounds as catalysts for the synthesis of biologically relevant compounds, biologically organometallic compounds, catalytic role of organometallic compounds in biomolecules, bioimaging probes, host-guest chemistry, the application of organometallic compounds in biosensors, and organometallic compounds as drugs.

Transition metal complexes are emerging promising alternatives to fluorescent organic compounds in various sensing applications. Some favourable properties of metal complexes enable them to be advantageous over organic dyes, such as stabilized redox states, kinetic lability of ligands and heavy atom effect. These properties enable certain biological functionalities. Among these functionalities of transition metal complexes, room temperature phosphorescence is particularly useful because of the involvement of excited triplet states and relative long decay lifetimes reaching µs level. And transition metal centers can cause ultrafast spin-orbit coupling, resulting in strong phosphorescence emission. Furthermore, the long emission lifetime can allow them to eliminate autofluorescence from interferences by the use of time-resolved techniques, resulting in increased sensitivity.

1.3 General introduction of luminescent cyclometalated Ir(III) complexes
Among the transition metal complexes, cyclometalated Ir(III) complexes are most widely explored for the development as sensing platforms due to their strong phosphorescence and tuneable emission by changing ligands.\textsuperscript{12} Generally, a cyclometalated Ir(III) complex contains three parts: core Ir atom, one ancillary (N\textsuperscript{N}) ligand and two cyclometalating (C\textsuperscript{N}) ligands, which adopts an octahedral geometry (Figure 1.1).\textsuperscript{13} Most explored C\textsuperscript{N} ligand is 2-phenylpyridine (ppy), in which ortho-position of the phenyl ring and its nitrogen form bonds to the metal center. Additionally, 1,10-phenanthroline and 2,2\textsuperscript{'}-bipyridine are most widely used N\textsuperscript{N} ligands. Important, this type of complex features in cyclometalation, which plays a key role in highly room-temperature quantum yield and its structural robustness.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{cyclometalatedIr.png}
\caption{General chemical structure of cyclometalated Ir(III) complex.}
\end{figure}

Ir(III) complex is generally synthesized through a two-step process. A chloride-bridged dinuclear Ir(III) dimer is firstly prepared through the Nonoyama reaction between Ir(III) salt and C\textsuperscript{N} ligands.\textsuperscript{14} The resulting Ir(III) dimer reacts with N\textsuperscript{N} ligand to yield the final phosphorescent
complex, which can be further prepared into different salt forms with the addition of different salts (Figure 1.2).

![Image](image_url)

**Figure 1.2.** The standard synthetic route of cyclometalated Ir(III) complex.

A cyclometalated Ir(III) complex produces at least two excited states upon the photoexcitation.\(^{12}\) The strong electronic effect of Ir atom renders the delocalization of frontier molecular orbitals of Ir(III) complexes over the whole molecule. The highest occupied molecular orbital (HOMO) of an Ir(III) complex is localized over the \(t_{2g}\) orbital of the Ir atom and the \(\pi\)-orbital of the \(C^N\) ligand, while the lowest-unoccupied molecular orbital (LUMO) is distributed widely at the \(N^N\) ligand and \(\pi^*\)-orbital of the \(C^N\) ligands.\(^{15,16}\) Photoexcitation between these frontier orbitals could promote at least two electronic transitions: a singlet metal-to-ligand charge-transfer (\(^1\text{MLCT};\ i.e.\ d\to\pi^*\)) transition state; a singlet ligand-centered (\(^1\text{LC};\ i.e.\ \pi\to\pi^*\)) transition state.\(^{17,18}\) Typically, the former is lower in energy than the latter. The heavy atom effect of Ir atom could promote ultrafast intersystem crossing from the \(^1\text{MLCT}\) transition state to \(^3\text{MLCT}\) transition state with the
rates over 100 fs.\textsuperscript{15} These internal conversion and intersystem crossing are much faster than fluorescence emitting, resulting in high-efficiency phosphorescence. Meanwhile, the hybrid nature of the triplet state gives the possibility that ligand modifications could tune photofunctions of the LC and MLCT transition states.\textsuperscript{19} Additionally, the emission properties of the complexes can be readily perturbed a change of the local environment of Ir(III) complexes. These photophysical modularities of Ir(III) complex are highly beneficial for perturbing the emission properties toward targeting a range of biological applications.

1.4 General strategies of Ir(III) complexes as chemosensors

In general, these are two main strategies employed to design Ir(III) complexes as chemosensors (Figure 1.3). One strategy utilizes intrinsically functional ligands to detect analytes, or involves the incorporation of the recognition unit of the analytes into the ligand without a spacer. The binding of these ligands with the targeted analytes usually changes the electronic structures of the ligands, thus causing the alternation of the emission properties of Ir(III) complexes. Another strategy involves the spacer linking the recognition unit to a ligand of Ir(III) complex. For the probe of the latter type, once the probe binds to the targeted biomolecule, its local environment turns to be more hydrophobic compared to the aqueous environment, leading to a signal change of the probe.
1.5 Ir(III) complexes as probes for cations, anions, small molecules and biomolecules

There are increasing reports about using luminescent Ir(III) complexes as probes for environmental analytes and biomolecules through the strategies above. It is a common strategy to incorporate a cation binding group such as a Lewis base or crown ether into a luminescent Ir(III) scaffold to develop cation chemosensors. For example, Lin and coworkers reported a crown ether-linked luminescent Ir(III) complex \([\text{Ir}(ppy)_2(\text{di-aza-phen})]^+\) (1), which aza-dithia-dioxa crown-ether on N^N ligand serves as an Ag^+ binder.\(^\text{20}\)

Complex 1 showed 3.4-fold enhancement upon the addition of Ag^+ (100 eq) in ACN/H_2O (1:1) around 595 nm. Although complex 1 showed good selectivity over many metal ions, Hg^{2+} as a strong interference restricts its
practical use. In another report, Nam and coworkers applied a cationic Ir(III) complex (2) to detect Zn$^{2+}$, which displayed turn-on yellow emission upon the binding of Zn$^{2+}$.\(^{21}\) Importantly, Zn$^{2+}$ binding in the cell would lead to an increase in the photoluminescence lifetime, which can be visualized using time-gated luminescence imaging.

**Figure 1.4.** Chemical structures of Ir(III) complexes 1 and 2 as chemosensors.

The emission properties of functional Ir(III) complexes can also be remarkably affected by the binding of anions to ligands, because of electronic effects or reactions between the ions and the ligands. Bian and co-workers reported a complex [Ir(ppy)$_2$(dmpp)]$^+$ (3) with a specific chromo-lumino-electro chemodosimeter for CN$^-$.\(^{22}\) CN$^-$ addition could destroy the conjugation in the N$^\alpha$N ligand of complex 3, thus changing the photophysical properties. In the presence of CN$^-$, the color of the solution changed from pink to colorless, and also produced a green emission band around 520 nm. Huang and coworkers developed a highly selective chemosensor for F$^-$ using bis-mesitylboryl group as a recognition unit. The
reaction between Ir(III) complex (4) and F\(^-\) induced solution color change from yellow to orange-red, which could be observed by naked-eyes.\(^{23}\) Additionally, the presence of around 2 equiv of F\(^-\) could completely quench the emission of complex 4, showing “turn-off” emission behavior.

**Figure 1.5.** Chemical structures of Ir(III) complexes 3 and 4 as chemosensors.

Ir(III) complexes are also widely employed as chemosensors for small molecules. As the triplet excited state of Ir(III) complexes can be effectively quenched by oxygen, so Ir(III) complexes have been widely explored to oxygen sensing. Ferna´ndez-Gutie´rrez and co-workers reported an oxygen-sensor films based on an Ir(III) complex (5).\(^{24}\) The film showed a quick quenching luminescence response against different oxygen concentrations. In addition, the sensing film showed the long-term stability and emission at 665 nm. Li and coworkers designed an Ir(III) complex-based probe for monitoring homocysteine (Hcy) and cysteine (Cys) based on cyclization reaction between the aldehyde group and the analytes.\(^{25}\) The probe displayed a red-shift from 547 nm to 586 nm upon the
addition with Hcy and Cys. Meanwhile, the results demonstrated that the probe could detect the changes of Hcy and Cys within living cells in a ratiometric behavior.

![Figure 1.6. Chemical structures of Ir(III) complexes 5 and 6 as chemosensors.](image)

Due to environmental-sensitive emission property, Ir(III) complexes have been employed to detect proteins by linking with a substrate. Lo and coworkers linked estradiol (the substrate of estrogen receptor α) to Ir(III) complex (7) as the probe for estrogen receptor α.\(^{26}\) The probe showed an approximate 3.3-fold emission enhancement against estrogen receptor α in phosphate-buffered saline, while the lifetimes of the complex increased as well after binding. Lo and coworkers also designed and synthesized Ir(III) complex (8) with biotin as avidin probe, which biotin displayed high affinity towards avidin.\(^{27}\) Complex 8 displayed 8.5-fold luminescence enhancement upon the addition of avidin. The avidin-binding properties of the complex have been confirmed by competitive assays using biotin. Additionally, the
emission lifetime of the complex 8 also increased upon the binding of avidin.

Figure 1.7. Chemical structures of Ir(III) complexes 7 and 8 as chemosensors.

In this thesis, I aim to utilize desirable characteristics of Ir(III) complexes to develop luminescent probes for a range of targets, such as metal ions, small molecules, and proteins. Meanwhile, several strategies will be explored to improve the biocompatibility of Ir(III)-based probes in order to explore their potential to be widely used as bioimaging probes. Hopefully, these low-cost, convenient and sensitive detection platforms could contribute to in-field study for environmental pollutions and point-of-care diagnosis.
Chapter 1

1.6 Reference


14. S. Park, C. D. Sunesh, H. Kim, H. Chae, J. Lee and Y. Choe, *Surface and


Chapter 2

Long-lived luminescent Ir(III)-based probe for selectively turn-on detection of Al$^{3+}$ ions

2.1 Introduction

Aluminium, the most abundant metal in the earth, do not naturally exist in nature. However, aluminium is widely used in many human activities e.g. industrial processes, causing the release of free aluminum ions (Al$^{3+}$) to the environment. Although low concentration of Al$^{3+}$ ions would not affect our health, high concentration of Al$^{3+}$ ions is toxic to the neuronal system and is highly associated with the pathology of Parkinson’s and Alzheimer’s disease, as well as causing damages to the organs.\(^1\), \(^2\) Importantly, the World Health Organization (WHO) reported that the concentration of Al$^{3+}$ ions in drinking water should be lower than 7.41 µM.

Traditional approaches for the detection of Al$^{3+}$ ions include inductively-coupled plasma mass spectrometry (ICP-MS) and atomic absorption spectroscopy (AAS)\(^3\),\(^4\). However, these techniques generally require complicated sample pre-treatment and preparation procedures. Moreover, these instruments usually non-portable, which greatly limited their application for \textit{in situ} detection of Al$^{3+}$ ions. As a consequence, organic chemosensors have become promising alternatives for sensitive and rapid detection of Al$^{3+}$ ions.

To date, a range of organic fluorophores have been applied for the detection
of Al\(^{3+}\) ions. However, to our knowledge, no transition metal complex-based chemosensor was available for Al\(^{3+}\) ions.\(^5\text{-}^{24}\) On the other hand, transition metal complexes-based chemosensors have been reported for the detection of a wide range of analytes due to their promising advantages.\(^25\text{-}^{27}\) In contrast to the organic dyes, transition metal complexes offer a long lifetime, which enables their emission to be recognized from the highly autofluorescent matrix, as well as a large Stoke shift, which could prevent self-quenching.\(^28\text{-}^{34}\) In this part, a novel Ir(III) complex-based Al\(^{3+}\) ions sensor that incorporates an Al\(^{3+}\) ions receptor, \(\sigma\)-phenolsalicylimine (PSI), in the N\(^-\)N ligand and contains two phenylpyridine (ppy) C\(^-\)N ligands have been reported (Scheme 2.1). The incorporation of PSI into luminescent Ir(III) complex could potentially allow the development of novel chemosensors for Al\(^{3+}\) ions in complicated biological media.

![Scheme 2.1. Schematic mechanism for Al\(^{3+}\) sensing by complex 1.](image)

2.2 Experimental section

2.2.1 Materials and general experiments

Chemicals, commercially available, were bought from Sigma Aldrich (St. Louis,
MO) or Precious Metals Online (Australia) and used without further purification. Luminescence spectra and emission lifetime for samples were recorded on a PTI TimeMaster C720 Spectrometer, HRMS was performed at the Mass Spectroscopy Unit of Hong Kong Baptist University. NMR spectra were measured on a Bruker Avance 400 spectrometer (\(^1\)H at 400 MHz, \(^{13}\)C at 101 MHz).

2.2.2 Synthesis of complex 1

Compound S1 was prepared based on a reported method.\textsuperscript{35} In a round bottomed flask, \(p\)-toluidine (1 eq), 1,10-phenanthroline-5,6-dione (1 eq), 4-hydroxy-3-nitrobenzaldehyde (1 eq) and ammonium acetate (10 eq) were mixed into acetic acid. Then the suspension was stirred and heated to reflux overnight. The resulting solution was then cooled to rt and poured into ice water, adjusted to pH 7 by \(\text{NH}_4\text{OH}\). The solution was then extracted by DCM for three times, the organic layer was then combined and dried over \(\text{Na}_2\text{SO}_4\). And the volatiles were removed \textit{in vacuo}. Silica gel column chromatography was carried out to purify the residue using the eluent (ethyl acetate: MeOH, 10:1) to yield the compound S1. Yield: 54%. \(^1\)H NMR (400 MHz; DMSO-\(d_6\)): 9.07 (dd, \(J = 4.0, 1.6\) Hz, 1H), 8.99 (dd, \(J = 8.4, 2.0\) Hz, 1H), 8.94 (dd, \(J = 4.4, 2.0\) Hz, 1H), 8.03 (d, \(J = 2.4\) Hz, 1H), 7.85 (dd, \(J = 8.0, 4.4\) Hz, 1H), 7.75 (dd, \(J = 8.8, 2.0\) Hz, 1H), 7.68 (d, \(J = 8.0\) Hz, 2H), 7.56 (d, \(J = 8.0\) Hz, 2H), 7.49 (dd, \(J = 8.4, 4.0\) Hz, 1H), 7.40 (dd, \(J = 8.4, 4.0\) Hz, 1H), 7.13 (d, \(J = 8.8\) Hz, 1H), 2.52 (s, 3H). \(^{13}\)C-NMR (400 MHz; DMSO-\(d_6\)): \(\delta\) 152.8, 149.8, 148.5, 147.5, 143.9, 143.6, 140.4, 136.4, 135.1, 134.9, 134.6,
131.2, 129.7, 128.5, 127.3, 126.6, 125.7, 123.8, 123.2, 122.5, 120.7, 119.3, 119.2, 21.0. HRMS: Calcd. for C_{26}H_{17}N_{5}O_{3}: m/z = 448.1410. Found: m/z = 448.1448. [M+H].
Chapter 2

1H NMR, 13C NMR and HRMS of S1.

Compound S2 was prepared according to a reported method. Compound S1 (0.224 g, 0.5 mmol) was added in EtOH (30 mL) with stirring for 1 h. Pd/C (0.20 g, 10% Pd) and 8 mL NH₂NH₂•H₂O were then added into the mixture, and the resulting suspension was refluxed overnight. The resulting reaction solution was filtered and evaporated to remove the solvent under reduced pressure condition. Silica gel column chromatography was carried out to purify the residue using the eluent (ethyl acetate: MeOH, 5:1, v/v) to obtain compound S2. Yield: 48%. 1H NMR (400 MHz; DMSO-d₆): 9.44 (s, 1H), 9.05 (dd, J = 4.4, 2.0 Hz, 1H), 8.95 (dd, J = 8.4, 2.4 Hz, 1H), 8.90 (dd, J = 4.4, 1.6 Hz, 1H), 7.83 (dd, J = 8.4, 4.4 Hz, 1H), 7.54-7.43 (m, 5H), 7.33 (dd, J = 8.4, 1.6 Hz, 1H), 7.11 (d, J = 2.4 Hz, 1H), 6.49 (d, J = 8.4 Hz, 1H), 6.39 (dd, J = 8.4, 2.4 Hz, 1H), 4.67 (s, 2H), 2.48 (s, 3H).

13C-NMR (400 MHz; DMSO-d₆): δ 153.2, 148.2, 147.1, 145.2, 143.7, 143.5, 139.7, 136.6, 135.3, 134.9, 130.8, 129.6, 128.6, 127.1, 126.2, 123.6, 123.4, 122.3,
121.0, 119.5, 117.5, 115.6, 113.5. HRMS: Calcd. for C_{26}H_{19}N_{5}O: m/z = 417.1590.

Found: m/z = 417.1579.
Complex S3 was prepared according to a reported method. The Ir(III) dimer \([\text{Ir}(ppy)_2\text{Cl}]_2\) (0.014 mmol) and compound S2 (0.03078 mmol) were added into the mixed solvent of 3 mL DCM and 3 mL MeOH. The mixed solution was stirred at rt overnight. Excess NH$_4$PF$_6$ solid was added into the reaction solution, the resulting solution was then stirred for further 30 min under rt. The volatiles were removed in vacuo and silica gel column chromatography was performed to purify the residue using the eluent (MeOH: DCM, 1:20, \(v/v\)) to afford compound S3 (brown solid). Yield: 65%. $^1$H NMR (400 MHz; DMSO-\(d_6\)): 9.56 (s, 1H), 9.23 (dd, \(J = 8.4, 1.2\ Hz, 1H\)), 8.28-8.20 (m, 3H), 8.11 (dd, \(J = 8.4, 4.8\ Hz, 1H\)), 8.03 (dd, \(J = 5.2, 1.6\ Hz, 1H\)), 7.96-7.84 (m, 4H), 7.74 (dd, \(J = 8.8, 5.2\ Hz, 1H\)), 7.60-7.45 (m, 7H), 7.12 (d, \(J = 2.4\ Hz, 1H\)), 7.12-6.90 (m, 6H), 6.51 (d, \(J = 8.4\ Hz, 1H\)), 6.42 (dd, \(J = 8.0, 2.4\ Hz, 1H\)), 6.25 (dd, \(J = 11.2, 7.2\ Hz, 1H\)), 4.73 (s, 2H), 2.48 (s, 3H). $^{13}$C NMR (400 MHz; DMSO-\(d_6\)): \(\delta 166.8, 155.2, 150.6, 150.2, \ldots\)
149.1, 149.0, 147.8, 145.8, 144.2, 144.1, 144.0, 140.5, 138.7, 136.8, 136.0, 134.5, 132.3, 131.2, 131.1, 130.3, 129.4, 128.5, 127.5, 127.4, 126.3, 125.9, 125.1, 123.9, 123.8, 122.3, 122.0, 120.2, 120.0, 117.7, 115.4, 113.6, 21.0. HRMS: Calcd. for $C_{48}H_{35}IrN_7O$: $m/z = 918.2532$. Found: $m/z = 918.2532$. 
1H NMR, 13C NMR and mass spectrum of compound S3.

Complex 1. A solution of 2-hydroxyl-benzaldehyde (1.9 mg, 0.0155 mmol) in EtOH was mixed with a solution containing complex S3 (15 mg, 0.0141 mmol) in 5 mL EtOH. The mixture was then refluxed for 5 h under nitrogen atmosphere. The solution was then cooled to 25 °C, and the solvent was evaporated to remove under reduced pressure. The brown product was obtained through precipitation from DCM and diethyl ether to yield complex 1. Yield: 68%. Overall yield: 11.5%.

1H-NMR (400 MHz; CDCl₃): δ 12.2 (s, 1H), 9.32 (d, J = 8.4 Hz, 1H), 8.40 (s, 1H), 8.23 (dd, J = 5.2, 1.2 Hz, 1H), 8.15 (dd, J = 4.8, 1.2 Hz, 1H), 7.90 (d, J = 8.0 Hz, 2H), 7.81-7.67 (m, 7H), 7.58 (d, J = 7.2 Hz, 1H), 7.52-7.47 (m, 2H), 7.42-7.31 (m, 7H), 7.10-7.02 (m, 3H), 7.00-6.91 (m, 5H), 6.86-6.83 (m, 1H), 6.41-6.38 (m, 2H), 2.55 (s, 3H). 13C-NMR (400 MHz; CD₃CN): 167.1, 160.5, 150.2, 150.1, 149.8, 149.2, 149.0, 148.4, 148.2, 144.7, 144.4, 143.8, 138.1, 136.2, 134.3, 133.2, 132.0, 131.3, 130.9, 130.0, 129.8, 128.1, 127.6, 126.1, 125.7, 124.5, 123.1, 122.2, 119.4, 119.0, 118.9, 20.3. HRMS: Calcd. for C₅₅H₃₀IrN₇O₂[M–PF₆]⁺: 1022.2794 Found:
1022.2756. Anal.: (C$_{55}$H$_{41}$IrN$_7$O$_2$PF$_6$) +1.5H$_2$O C, H, N: calcd. 55.23, 3.71, 8.20; found 55.07, 3.33, 8.32.
25

\[ ^1H \text{ NMR}, \ ^{13}C \text{ NMR and mass spectrum of complex } 1.\]

2.2.3 The detection of Al\(^{3+}\) ions

Complex 1 was dissolved into ACN as a stock solution with a concentration of 10 mM. 20 µM complex 1 in ACN was first prepared using stock solution. In the mixture of 995 µL ACN containing 20 µM complex 1 and 5 µL H\(_2\)O, Al\(^{3+}\) ions were added with indicated concentrations. A PTI QM-4 spectrofluorometer was then used to obtain luminescence spectra, while a Cary UV-300 spectrophotometer was applied to record absorption spectra.

2.2.4 Live cell imaging assay

In cover glass-bottom confocal dishes, HepG2 cells were seeded with a density of \(1 \times 10^6\) cells per well. The cells were treated with 10 µM complex 1 for 1 h under 37 °C. Next, these cells were washed by using PBS for three times, followed by the treatment of vehicle control or Al\(^{3+}\) (100 µM) for a further 30 min at 37 °C.
Finally, a Leica TCS SP8 confocal microscope was used to take the images of the cells using a 20 × objective lens.

### 2.3 Results and discussion

The photophysical profiles of Ir(III) complex in different solvent environments can be fine-tuned by modifying their N^N or C^N ligands. Therefore, we synthesized an Ir(III) complex incorporating a PSI into its N^N ligand, as well as containing two ppy as C^N ligands (Figure 2.1). When designing an Ir(III) complex-based chemosensor, the LUMO of complex 1 should mainly depend on the PSI-containing ligand. \(^{38-40}\) Consequently, the binding of Al\(^{3+}\) ions at PSI would significantly affect the LUMO of the metal-to-ligand charge-transfer (MLCT) state of the complex, substantially altering the absorption and emission properties of the complex. Therefore, the complex may serve as a luminescent probe for Al\(^{3+}\) ions.

Complex 1 was synthesized and prepared according to the modified literature procedures (Figure 2.1). Intermediate \textbf{S1} was generated from the multicomponent Radziszewski reaction with 1,10-phenanthroline-5,6-dione as the starting material, which went through in two stages. The dicarbonyl, ammonia produced from \(\text{NH}_4\text{OAc}\), and \(p\)-toluidine condensed to give a diimine derivative, which then condenses with the aldehyde to give desired \textbf{S1}. \(^{41}\) Compound \textbf{S1} would be reduced in the presence of Pd/C and \(\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}\), leading to the generation of compound \textbf{S2} in moderate yield. The resulting \textbf{S2} reacted with 0.5 equivalent of
the organometallated dimer [Ir(ppy)$_2$Cl]$_2$ to produce compound S3 in 65% yield, followed by anion exchange with NH$_4$PF$_6$. Subsequent condensation between compound S3 and 2-hydroxyl-benzaldehyde under standard reaction conditions yielded complex 1 with a yield of 45%. These compounds were all structurally characterized using $^1$H NMR, $^{13}$C NMR spectroscopy, elemental analysis, and HRMS.

![Figure 2.1](image-url)

**Figure 2.1.** Synthetic route of complex 1. Reagents and conditions: a) NH$_4$OAc, acetic acid, reflux, overnight; b) Pd/C (10%), NH$_2$NH$_2$•H$_2$O, EtOH, reflux, overnight; c) Ir(III) dimer [Ir(ppy)$_2$Cl]$_2$, MeOH:DCM = 1:1, rt, overnight, then NH$_4$PF$_6$; d) 2-hydroxyl-benzaldehyde, EtOH, reflux, 5 h.

The photophysical properties of complex 1 were also studied (Figure 2.2).
exhibits a lifetime of ca. 4.2 µs (Table 2.1), which is similar to the typical luminescent transition metal complexes, while organic dyes generally display lifetimes in nanoseconds. The result shows that the development of transition metal complexes as chemosensors is an attractive alternative, as the long-lived emission could allow their luminescence to be distinguished from a highly autofluorescence biological media by using a time-resolved luminescence technique. Moreover, upon excitation at 350 nm, 1 displayed a maximum emission wavelength at 573 nm, with 230 nm of Stokes shift.

![Figure 2.2](image)

**Figure 2.2.** UV-Vis absorption spectra of 5 µM complex 1 in ACN.

<table>
<thead>
<tr>
<th>Quantum Yield</th>
<th>λ_{emi} / nm</th>
<th>Lifetime / µs</th>
<th>UV-Vis absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0082</td>
<td>573</td>
<td>4.201</td>
<td>λ_{abs} / nm (ε / dm^3 mol^{-1} cm^{-1})</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>259 (7.06×10^4), 353 (2.38×10^4)</td>
</tr>
</tbody>
</table>

Note: All were measured in ACN.
The luminescence response of complex 1 to Al\(^{3+}\) ions was also investigated. Low luminescence intensity was exhibited by complex 1 in the absence of Al\(^{3+}\) ion. Encouragingly, 1 exhibited enhanced luminescence in the presence of Al\(^{3+}\) ions. A time-course experiment reveals that the luminescence of complex 1 reached a plateau in 5 min in the presence of 3 µM of Al\(^{3+}\) ions (Figure 2.3), while luminescence was not observed in the absence of 1 (Figure 2.4). Considering there is a number of small organic probes with good aqueous solubility for Al\(^{3+}\) have been reported,\(^{42-47}\) we aim at studying the luminescence response of 1 to Al\(^{3+}\) in a different ratio of water. As shown in Figure 2.5, complex 1 performs the best in the system containing 5% of water, while an inferior luminescence enhancement was detected in 10% of water. The response of 1 to Al\(^{3+}\) ions in other solvents with 5% H\(_2\)O such as DMSO and DMF was also investigated. We found that ACN could result in the highest luminescence enhancement among these solvents (Figure 2.6). Furthermore, the luminescence enhancement for 1 to Al\(^{3+}\) ions was found to be pH independent in the pH range of 4–9. (Figure 2.7). Meanwhile, upon the addition of increasing concentration of Al\(^{3+}\) ions, the luminescence of 1 increased accordingly and the luminescence was saturated at 45 µM of Al\(^{3+}\) ions, displaying a ca. 13.5-fold luminescence enhancement (Figure 2.9). The binding constant \(1.145 \times 10^4\) M\(^{-1}\) was also calculated based on the Benesi-Hildebrand equation.\(^{48,49}\)
Figure 2.3. Time course of luminescence enhancement of 20 µM complex 1 in the presence of 3 µM Al\(^{3+}\) ions in ACN at 25 °C.

Figure 2.4. Luminescence spectra of 20 µM Al\(^{3+}\) with or without the addition of 20 µM complex 1 in ACN.
Figure 2.5. Luminescence response of 20 µM complex 1 to 20 µM Al\textsuperscript{3+} in various ACN:H\textsubscript{2}O mixtures.

![Figure 2.5](image)

Figure 2.6. Luminescence enhancement of 20 µM complex 1 with the addition of 20 µM Al\textsuperscript{3+} in different organic solvents with 5% H\textsubscript{2}O.

![Figure 2.6](image)

Figure 2.7. pH Effect on the relative luminescence enhancement of 20 µM complex 1 towards 20 µM Al\textsuperscript{3+}.

![Figure 2.7](image)

Furthermore, the UV-Vis absorbance of 1 was found to increase in the range
of 230–315 nm in the presence of Al\(^{3+}\) ions, while the absorption at 380 nm decreased (Figure 2.8). Furthermore, the limit of detection (LOD) for 1 was estimated to be 1 µM with a signal-to-noise ratio of 3, while reaching a linear relationship (\(R^2 = 0.99\)) in the range of 1–30 µM Al\(^{3+}\) ions (Figures 2.9a and b). The results show that 1 is capable of detecting Al\(^{3+}\) ion with sensitivity comparable to the reported chemosensors. Significantly, the LOD of 1 was lower than the safe limit in drinking water according to the WHO. For better comparison, sensing performances for recently developed chemosensors for the detection of Al\(^{3+}\) ions are summarized in Table 2.2. Importantly, the luminescence difference of 1 in the absence and presence of Al\(^{3+}\) ions could be visually observed under UV illumination at 365 nm (Figure 2.9c).

![Figure 2.8. UV-Vis absorption spectra of 20 µM complex 1 in the presence of different concentrations of Al\(^{3+}\) ions ranging from 0 to 40 µM in ACN](image-url)
Figure 2.9. (a) Luminescence enhancement of 20 µM complex 1 to different concentrations of Al\(^{3+}\) ions from 0 to 55 µM in ACN. (b) The linear relationship between the luminescence of complex 1 and the concentration of Al\(^{3+}\). (c) Photograph images of 20 µM complex 1 with (right) or without (left) the addition of 40 µM Al\(^{3+}\) ions under UV illumination at 365 nm. (d) Luminescence response of complex 1 to Al\(^{3+}\) (20 µM) with the addition of EDTA (20 µM).

Table 2.2. Comparison of the recent chemosensors for the detection of Al\(^{3+}\) ions.

<table>
<thead>
<tr>
<th>Target</th>
<th>Detection range</th>
<th>Detection limit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al(^{3+})</td>
<td>1–30 µM</td>
<td>1 µM</td>
<td>This study</td>
</tr>
<tr>
<td>Al(^{3+})</td>
<td>1–20 µM</td>
<td>1 µM</td>
<td>15</td>
</tr>
<tr>
<td>Al(^{3+})</td>
<td>1–12 µM</td>
<td>1 µM</td>
<td>18</td>
</tr>
</tbody>
</table>
The interaction of Al$^{3+}$ ions with the PSI-conjugated N$^\wedge$N ligand could alter the MLCT state of complex 1, leading to a “switch on” luminescence of the probe. The relationship between the luminescence enhancement of complex 1 and the coordination of Al$^{3+}$ ions to complex 1 was also validated. Considering the ability of ethylenediaminetetraacetic acid (EDTA) to sequester metal ions, EDTA was introduced to deprive the Al$^{3+}$ ions of Al$^{3+}$-coordinated 1. Encouragingly, the luminescence of the 1–Al$^{3+}$ substantially decreased upon addition of EDTA (Figure 2.9d). However, upon the further addition of Al$^{3+}$ ions at a final
concentration exceeding the loaded EDTA, complex 1 restored its luminescence as expected.

To further demonstrate the long-lived properties of 1, time-resolved emission spectra (TRES) measurement was performed. In this work, coumarin (Cm) was introduced to the solution contain 1 as sources of interfering fluorescence stimulating the autofluorescence background in typical biological samples. In steady-state, Cm exhibits a strong fluorescence emission peak at 420 nm, with a tail extending to 650 nm. The luminescence peak of complex 1 is strongly impaired by the tail and the fold enhancement of 1 was barely evident (Figure 2.10a). Encouragingly, 1 was clearly observed upon delaying the time for receiving luminescence signal after excitation. As the time gate was set after the fluorescence decay of Cm, the fluorescence emission of Cm was eliminated and the emission peak of 1 had become more evident (Figure 2.10b). Protoporphyrin IX (PPIX) was also employed as an interfering specie in TRES experiment. Consistent with the results with Cm as a fluorescent agent, PPIX exhibits strong fluorescence between 630 and 710 nm. As a result, the luminescence of 1 heavily overlaps with the fluorescence of PPIX, and the fold-enhancement of 1 was difficult to determine (Figure 2.10c). Notably, upon setting the time gate to the time after the complete fluorescence decay of PPIX, the luminescence of complex 1 was clearly observed. As a result, the fluorescence of PPIX was not observed, while the luminescence of complex 1 was dominant (Figure 2.10d). These data demonstrate the promising advantage of complex 1 over typical organic
fluorophores. The long-lived emission of 1 could be distinguished by TRES, showing the potential in the applications in the biological samples that are highly-autofluorescent.

Figure 2.10. Time-resolved luminescence spectra of complex 1 with or without the addition of Al^{3+} ions (25 µM) in the presence of Cm (a, b) and protoporphyrin IX (c, d).

To investigate the selectivity of the 1 toward other metal ions, we introduced 5-fold excess of other metal ions (Cs^+, Li^+, Na^+, K^+, Pb^{2+}, Ba^{2+}, Cu^{2+}, Fe^{3+}, Ag^+, Cd^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Cr^{3+} and Mn^{2+}) in 1 (Figure 2.11a). Encouragingly, the results reveal that 1 only shows significant luminescence enhancement upon the addition of Al^{3+} ions. Other common metal ions in the physiological environments, including Na^+ and K^+, had no significant effect on luminescence of complex 1,
nor other Group I ions including Cs\(^+\) and Li\(^+\). Only a slight luminescence response was exhibited by 1 with the addition of Ag\(^+\), Ba\(^{2+}\), Pb\(^{2+}\) and Cr\(^{3+}\) ions, presumably attributed to the slight interaction between these cations and the PSI moiety that have been reported in the literature.\(^{15}\) Taken together, these verify the selectivity of 1 for Al\(^{3+}\) ions over other interfering ions. A competition experiment was also employed to study the response of 1 to 20 \(\mu\)M of Al\(^{3+}\) ions with the addition of other interfering metal ions. Encouragingly, the luminescence enhancement of complex 1 in the presence of Al\(^{3+}\) ion was not significantly affected upon the addition of the same concentration of other interfering cations (Figure 2.11b). The result suggests that 1 could potentially be used to detect Al\(^{3+}\) ions in real samples containing a certain concentration of interfering metal ions.

![Figure 2.11](image)

**Figure 2.11.** (a) Luminescence enhancement of complex 1 (20 \(\mu\)M) with Al\(^{3+}\) (25 \(\mu\)M) or 5-fold excess of other interfering metal ions, such as Na\(^+\), Cs\(^+\), K\(^+\), Li\(^+\), Ag\(^+\), Ba\(^{2+}\), Mn\(^{2+}\), Pb\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\), Cr\(^{3+}\) and Fe\(^{3+}\) ions. (b) Luminescence enhancement of complex 1 (20 \(\mu\)M) with the addition of both 20 \(\mu\)M of Al\(^{3+}\) and Cs\(^+\), Na\(^+\), K\(^+\), Li\(^+\), Ag\(^+\), Ba\(^{2+}\), Mn\(^{2+}\), Pb\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\), Cr\(^{3+}\) and Fe\(^{3+}\) ions.
The application of complex 1 in biological samples was also investigated by repeating the calibration experiment of 1 in 2.5% (v/v) serum using the time-resolved technique. Notably, a ca. 4-fold luminescence enhancement was obtained after adding 10 µM of Al$^{3+}$ (Figure 2.12) to the system. The result suggests that 1 could potentially be employed for detecting Al$^{3+}$ ion in biological samples. At last, the imaging performance of complex 1 for Al$^{3+}$ ions in living cells was studied. Upon treating the cells with Al$^{3+}$ ions (100 µM) in the culture medium at 37 °C for 30 min, yellow emission was observed in the cells (Figure 2.13). These results demonstrate the membrane permeability of 1 and its ability to detect intracellular Al$^{3+}$ ions.

![Figure 2.12. Time-resolved luminescence spectra of 1 in the solution containing 2.5% serum with or without the addition of Al$^{3+}$ ions (10 µM).](image)
Figure 2.13. The imaging of Al\textsuperscript{3+} ions in HepG2 cells using complex 1. HepG2 cells were first treated with 1 (10 µM) for 1 h, followed by the treatment with vehicle control or Al\textsuperscript{3+} ions (100 µM) for 30 min at 37 °C. The excitation wavelength was set at 405 nm. The scale bar is 100 µm.

2.4 Conclusion

In summary, we herein reported a luminescent Ir(III) complex 1 as a switch-on chemosensor for Al\textsuperscript{3+} ions. Complex 1 consists of two ppy C\(^\text{N}\) ligands and an Al\textsuperscript{3+} receptor-linked N\(^\text{N}\) ligand. Complex 1 achieved a maximum 13.5-fold luminescence enhancement at 573 nm upon the addition of Al\textsuperscript{3+} ions, and reaching a linear detection range at 1–30 µM and a detection limit of 1 µM. The result demonstrates its ability to detect Al\textsuperscript{3+} ions above the safety level in drinking water as mandated by the WHO. 1 also exhibits promising selectivity for Al\textsuperscript{3+} ions over
other metal ions, such as Cs\(^+\), Na\(^+\), K\(^+\), Li\(^+\), Ag\(^+\), Ba\(^{2+}\), Pb\(^{2+}\), Cu\(^{2+}\), and Fe\(^{3+}\). To the best of our knowledge, 1 is the first Ir(III) complex-based chemosensor for the detection of Al\(^{3+}\) ions. In contrast to the typical organic chemosensors, 1 displays a long-lifetime luminescence and large Stokes shift. These advantages allow 1 to detect Al\(^{3+}\) ions in those high autofluorescence media using TRES and potentially allow 1 to be employed in biological samples.
2.5 Reference


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Chapter 2

American Chemical Society, 2004, 126, 14129.


Chapter 3

Luminescent Ir(III) complex for visualizing H$_2$S and hypoxia in living zebrafish

3.1 Introduction

Reactive sulfur species (RSS) are sulfur-containing chemically reactive molecules that are critical to many biological processes, such as maintaining redox homeostasis, metabolism regulation, and cell signaling.\textsuperscript{1} Among the RSS, hydrogen sulfide (H$_2$S) is the most studied, which is one of the well-known endogenous gaseous molecules as both carbon monoxide (CO) and nitric oxide (NO).\textsuperscript{2} In our body, L-cysteine (L-Cys) is the main source of H$_2$S, which produces H$_2$S in the presence of enzymes such as cystathionine gamma-lyase or cystathionine beta-synthase. H$_2$S has been demonstrated as an important signaling molecule in human systems, such as immune system,\textsuperscript{3} cardiovascular system,\textsuperscript{4-6} and central nervous system.\textsuperscript{7,8} Meanwhile, H$_2$S plays an important role in other biological processes, such as the regulation of kidney functions,\textsuperscript{9} maintaining homeostasis of stem cells,\textsuperscript{10} as well as the activation of T cells.\textsuperscript{11} Moreover, the abnormal H$_2$S levels have also been associated with a range of diseases including chronic kidney disease,\textsuperscript{12} Alzheimer's disease,\textsuperscript{13} Parkinson's disease,\textsuperscript{14} and high blood pressure.\textsuperscript{15}

Considering the physiological roles of H$_2$S, a convenient, sensitive, selective, and biocompatible detection tool are of high importance. Traditional
instrument-based methods for the detection of $\text{H}_2\text{S}$, such as spectrophotometric determination,\textsuperscript{16} gas-chromatographic assays,\textsuperscript{17} and electrochemical analysis,\textsuperscript{18} and gas chromatography-mass spectrometry,\textsuperscript{19} have been widely used. However, these established methods generally suffer from tedious sample pre-treatment procedures, high cost, and time-consuming sample preparation procedures. Furthermore, these assays are not applicable to the detection of $\text{H}_2\text{S}$ in living cells or organisms in an invasive manner. To circumvent these problems, various small-molecule dyes have been reported for monitoring $\text{H}_2\text{S}$ in the living systems in recent years.\textsuperscript{20-25} In particular, reaction-based probes for $\text{H}_2\text{S}$ detection probes, which exhibit low background have attracted many interests.\textsuperscript{26-28} However, these probes are generally limited by their inherent drawbacks, including low photostability and small Stokes shift.

On the other hand, hypoxia has been identified as an important hallmark of cancers.\textsuperscript{29-31} It is reported that hypoxia can accelerate malignant progression and increase the chances of metastasis in cancers, as well as hamper the therapeutic effect of radiation therapy.\textsuperscript{32} Furthermore, hypoxia in tumors also results in resistance to chemotherapy, due to the enhanced genetic instability, pH variations, and low accessibility to oxygen.\textsuperscript{33, 34} Considering the role of hypoxia in solid tumors, a range of assays for tumor hypoxia have been developed, including hypoxia-related protein assays,\textsuperscript{35} blood flow velocity assays,\textsuperscript{36} and the oxygen sensors.\textsuperscript{37}

Compared to traditional small molecule-based fluorescence probes,\textsuperscript{38-42}
considerable attention has been paid on the biosensing, chemosensing, and bioimaging applications of transition metal complexes. Transition metal complexes possess many attractive advantages due to their promising photophysical properties, such as tunable luminescence emission, long-lived emission lifetime, high photostability, and large Stokes shift. To our knowledge, no Ir(III) complex is reported to detect H$_2$S and hypoxic conditions in the living systems.

In this work, a new luminescent Ir(III) complex was designed and synthesized with the dual functions of H$_2$S detection and hypoxia measurement. The complex was proposed based on those structural modifications on the 1,10-phenanthroline (phen) N$^\text{N}$ ligand that affect the luminescence properties of the phen containing Ir(III) complexes due to non-radiative decays to the relaxation of complexes’ excited state. For example, strong electron-withdrawing groups such as nitro group can quench the luminescence of Ir(III) complexes. Therefore, we reasoned that a nitro group at a phen may serve as a luminescence quencher for Ir(III) complexes. For the two conjugated C$^\text{N}$ ligands, a novel Ir(III) complex [Ir(4-Fphq)$_2$(5-nitrophen)]$^+$ (1) (where 4-Fphq = 2-(4-fluorophenyl)quinoline, 5-nitrophen = 5-nitro-1,10-phenanthroline) was prepared according to a previously reported method. The ligand 4-Fpq was prepared according to a classical C-C coupling reaction between (4-fluorophenyl)boronic acid and 2-bromoquinoline. A complex containing an amino group instead of the nitro group on the phen was also synthesized for comparative analysis, which is
[Ir(4-Fpq)\(_2\)(5-aminophen)]^+ \quad (2) \quad \text{(where 5-aminophen = 5-amino-1,10-phenanthroline)}.

\(^1\)H NMR, \(^{13}\)C NMR, and HRMS were used to confirm their structures. Purity investigation of 1 was performed by HPLC. We expected that the presence of H\(_2\)S or hypoxic condition could reduce 1 into 2, along with a detectable and observable luminogenic enhancement (Scheme 3.1). Therefore, 1 can serve as a luminogenic sensor for H\(_2\)S in the living systems, as well as a bioimaging probe of hypoxic living cells and organisms.

\[ \begin{align*}
1 & \quad \text{in hypoxia or in hypoxia} \\
1 & \quad \text{H\(_2\)S} \\
2 & \quad \text{NH}_3
\end{align*} \]

\textbf{Scheme 3.1.} Schematic mechanism for H\(_2\)S and hypoxia detection by complex 1.

3.2 Experimental section

3.2.1 Materials and general experimental

Chemicals, commercially available, were bought from Sigma Aldrich (St. Louis, MO) or Precious Metals Online (Australia) and used without further purification. Luminescence spectra and emission lifetime for samples were recorded on a PTI TimeMaster C720 Spectrometer, HRMS was recorded at the Mass Spectroscopy Unit at Hong Kong Baptist University, Hong Kong. NMR experiments were
performed on a Bruker Avance 400 spectrometer (\(^1\)H at 400.1 MHz, \(^{13}\)C at 101 MHz). Unless specified, the H\(_2\)S mentioned in this manuscript was produced from Na\(_2\)S.

### 3.2.2 H\(_2\)S detection

Complex 1 was dissolved into ACN as a stock solution with a concentration of 5 mM. Complex 1 and H\(_2\)S were added into EtOH with a final concentration of 10 \(\mu\)M for the complex (10 \(\mu\)M) and indicated concentrations for H\(_2\)S, which reached a final volume of 500 \(\mu\)L. A PTI QM-4 spectrofluorometer was then used to record luminescence spectra of samples.

### 3.2.3 Stability experiment

5 mM complex 1 in DMSO-\(d_6/\)D\(_2\)O \((v/v = 9:1)\) was kept for was seven days at rt, which was measured by \(^1\)H NMR each 24 h.\(^58\) The \(^1\)H NMR spectra were determined on a Bruker Avance 400 spectrometer.

### 3.2.4 Solubility experiment

Based on a previously reported solubility measurement method, the solubility of complexes was determined.\(^59\) A stock solution of 10 mM in DMSO was first prepared, which was subsequently diluted with concentrations \((5 \times 10^{-7} - 5 \times 10^{-4} \text{ mol/L})\) in HEPES (HEPES/ACN, 8/2, pH 7.0). A saturated solution was also prepared and all samples were measured by UV/Vis absorption spectroscopy from
230 to 600 nm.

3.2.5 Purity experiment

An Agilent 1200 HPLC system was also applied to determine the purity of the complex. Milli-Q H$_2$O (0.1% v/v TFA) served as mobile phase A, while ACN (0.1% v/v TFA) served as mobile phase B. The mobile phase gradient started from 10% ACN, increased 10–30% ACN from 5 min to 15 min, 30–90% ACN from 5 min to 15 min and ended up as a 95% ACN over a time course of 25 min. UV absorbance was monitored at 254 and 280 nm, respectively, and 1.0 mL/min was set as the flow rate. Additionally, an Agilent C18 column (4.6 mm × 250 mm, 5 µm) was used.

3.2.6 Cell imaging of H$_2$S

For exogenous H$_2$S imaging, the H$_2$S was produced from Na$_2$S. In cover glass-bottom confocal dishes, HeLa cells were seeded. The cells were treated with complex 1 at the indicated concentration for 1 h under 37 °C. These cells were washed using PBS for three times, followed by the treatment of vehicle control or H$_2$S for a further 1 h at 37 °C. The cells were subsequently washed by PBS (three times) and fresh medium. Finally, a Leica TCS SP8 confocal microscope was used to take the images of the cells using a 40 × objective lens. The excitation wavelength was set at 405 nm.

For endogenous H$_2$S imaging, HeLa cells were treated with 1 at the indicated
concentration for 1 h under 37 °C, Cys or GSH were subsequently added at the indicated concentrations into the growth medium for 1 h. Finally, a Leica TCS SP8 confocal microscope was used to take the images of the cells using a 40 × objective lens. The excitation wavelength was set at 405 nm.

### 3.2.7 Zebrafish imaging of H₂S

Zebrafish embryo preparation was performed as previously described. Zebrafish were kept separately with a 14 h light/10 h dark cycle under standard conditions. The generation of zebrafish embryos was from natural pair-wise mating (3–12 months old), followed by being raised in embryo water at 28.5 °C. 3 days old zebrafish embryos were pre-incubated with 20 µM complex 1 at 72 hpf. Na₂S was used as a source of H₂S. Zebrafish embryos were then treated by vehicle control or indicated concentrations of H₂S for a further 1 h at 37 °C and luminescence images of the embryos were taken at 74 hpf. A luminescence microscope was then used to take luminescence images.

### 3.2.8 Hypoxic cell imaging

In cover glass-bottom confocal dishes, HeLa cells were seeded. The cells were treated with the indicated concentration of complex 1 for 12 h at 37 °C under hypoxia. The cells were subsequently washed by PBS (three times) and fresh medium. A Leica TCS SP8 confocal microscope was used to take the images of the cells using a 40 × objective lens. The excitation wavelength was set at 405 nm.
3.2.9 Hypoxic zebrafish imaging

Zebrafish were kept separately with a 14 h light/10 h dark cycle under standard conditions. The generation of zebrafish embryos was from natural pair-wise mating (3–12 months old), followed by being raised in embryo water at 28.5 °C. 3 days old zebrafish embryos were incubated with 20 µM complex 1 with or without the addition of 20 µM complex 1 at 72 hpf. Subsequently, the cells were washed by PBS (three times) and fresh medium. A confocal luminescence microscope was used to take luminescence images.

3.2.10 Cytotoxicity assay

In a 96-well culture microplate, HeLa and LO2 cells were first seeded with the density of 5000 cells per well, respectively, followed by the incubation of 72 h at 37 °C. Complex 1 at different concentrations (0.01−100 µM) was added into each well, followed by the incubation in a humidified incubator for three days at 37 °C, 5% CO₂, 95% air. 1 mg/mL MTT reagent was added for 4 h. 100 µL of DMSO was used to replace the medium, followed by the incubation for 10 min at rt with shaking. A microplate reader was used to measure color intensity at 570 nm. The surviving cells curve was determined after exposure to complex 1 for three days for the IC₅₀ value of complex 1.

3.2.11 Nitroreductase (NTR) activity assay
The experiment was performed according to the human NTR ELISA kit manual. In 6 well plates, HeLa cells were seeded with a density of $1 \times 10^6$ cells. Cells were incubated for the indicated time at 37 °C. Cells lysates were collected and 40 µL from each sample was added to a well in a 96-well plate. 100 µL HRP-conjugate reagent was then added, followed by the incubation for 1 h at 37 °C. Chromogen solution A (50 µL) and B (50 µL) were added into wells with gentle mixing, and the wells were incubated for 15 min at 37 °C, followed by adding stop buffer. The plate was read at 450 nm using a microtiter platereader within 15 min of adding the stop buffer.

3.2.12 Synthesis of complex 1

**Synthesis of 4-Fpq.** Ligand 4-Fpq was synthesized using a modified literature method. 61 2-bromoquinoline (1 eq), tetrakis(triphenylphosphine)palladium(0) (0.05 eq), (4-fluorophenyl)boronic acid (1.2 eq), and potassium carbonate (2 eq) were mixed into EtOH/H$_2$O (95:5, v/v) in a sealed tube. The mixture was stirred and refluxed overnight. The reaction solution was then cooled to rt, and it was poured into water. DCM was used to extract the resulting solution three times, the combined organic layer was washed by sat. NaCl solution and dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated to give crude product under reduced pressure. Silica gel column chromatography was carried out to purify the residue (eluent, hexane:ethyl acetate: 50:1–20:1, v/v) to obtained ligand 4-Fpq. Yield: 75%.

$^1$H NMR (400 MHz, CDCl$_3$-d) $\delta$ 8.23 (d, $J = 8.6$ Hz, 1H), 8.21 – 8.13
(m, 3H), 7.87 – 7.81 (m, 2H), 7.74 (t, $J = 8.4$ Hz, 1H), 7.54 (t, $J = 8.0$ Hz, 1H), 7.25 – 7.17 (m, 2H). MALDI-TOF-HRMS calcd for C$_{15}$H$_{10}$FN [M – PF$_6$]$^+$/224.0881. Found: 224.0867.

**Synthesis of [Ir$_2$(4-Fpq)$_4$Cl$_2$] dimer.** Cyclometalated dichloro-bridged dimers [Ir$_2$(4-Fpq)$_4$Cl$_2$] (where 4-Fpq = 2-(4-fluorophenyl)quinolone) were prepared based on our previous method. IrCl$_3$$\cdot$H$_2$O (1 eq) and cyclometallated ligand 4-Fpq (2.1 eq) was added into the mixed solvent 2-methoxyethanol/H$_2$O, and the mixture was refluxed overnight under a nitrogen atmosphere. The resulting mixture was cooled to rt. And the precipitate was filtered and washed by H$_2$O and diethyl ether to afford the desired dimer Ir$_2$(4-Fpq)$_4$Cl$_2$, yield: 82%.

**Synthetic route of [Ir(4-Fpq)$_2$(N$^N$)]PF$_6$ complexes.** The complexes were prepared according to a modified reported procedure.$^{57}$ In brief, [Ir$_2$(4-Fpq)$_4$Cl$_2$] (1 eq) and corresponding N$^N$ ligands (2.1 eq) ligands were dissolved into a mixed solvent of DCM:MeOH, the resulting mixture was refluxed overnight. The reaction solution was then cooled to rt, and undissolved solid was filtered to remove. Excess NH$_4$PF$_6$ solid was added into the filtrate, and the solution stirred for another 30 min at rt. The resulting solution was evaporated to precipitate the crude product. The solid was filtered and washed by H$_2$O and diethyl ether. The resulting solid was then dissolved into acetone and precipitated by adding diethyl ether again, finally desired complexes were filtered to obtain.

**Complex 1:** (Yield: 51%, overall yield: 31.4%) HPLC purity: ≥ 95%. $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ 9.22 (d, $J = 8.8$, 1H), 9.13 (s, 1H), 9.11 – 8.93 (m, 3H),
8.60 – 8.49 (m, 4H), 8.45 – 8.41 (m, 2H), 8.31 – 8.25 (m, 2H), 7.83 (d, J = 8.0 Hz, 2H), 7.37 – 7.21 (m, 4H), 7.06 (t, J = 8.8 Hz, 2H), 6.94 (t, J = 8.7 Hz, 2H), 6.33 (d, J = 9.5 Hz, 2H). $^{13}$C NMR (100 MHz, acetone) δ 170.18, 170.12, 165.63, 153.82, 153.30, 153.14, 151.57, 148.76, 148.26, 147.74, 145.77, 143.57, 142.00, 141.68, 136.63, 132.07, 132.01, 131.08, 130.99, 130.29, 130.27, 129.21, 129.15, 128.97, 128.62, 127.94, 127.73, 124.67, 124.63, 124.54, 121.34, 121.16, 119.29, 111.75, 111.52. MALDI-TOF-HRMS calcd for C$_{42}$H$_{25}$F$_2$IrN$_5$O$_2$ [M – PF$_6$]$^+$: 862.1603; found: 862.1642.

HPLC trace for complex 1.

Complex 2: (Yield: 46%) $^1$H NMR (400 MHz, acetone-$d_6$) δ 8.92 (d, J = 8.5 Hz, 1H), 8.75 (d, J = 5.1, 1.3 Hz, 1H), 8.51 (s, 3H), 8.41 (t, J = 3.2 Hz, 1H), 8.39 (t, J = 5.6 Hz, 1H), 8.33 (d, J = 5.0 Hz, 1H), 8.27 (d, J = 8.0 Hz, 1H), 8.04 (dd, J = 8.5, 5.1 Hz, 1H), 7.85 (d, J = 4.9 Hz, 1H), 7.82 (d, J = 4.6 Hz, 1H), 7.78 (dd, J = 8.3, 5.0 Hz, 1H), 7.37 – 7.28 (m, 4H), 7.06 – 6.99 (m, 3 H), 6.93 (q, J = 7.5 Hz, 2H), 6.30 (t, J = 12.0 Hz, 2H), 6.25 (d, J = 6.9 Hz, 1H). $^{13}$C NMR (100 MHz, acetone)
δ 170.28, 149.69, 148.30, 145.37, 144.78, 141.40, 136.26, 134.38, 133.83, 131.74, 130.82, 130.17, 130.11, 128.52, 127.64, 127.39, 126.48, 124.86, 124.76, 124.69, 121.04, 119.12, 119.09, 103.62. MALDI-TOF-HRMS calcd for 
\[\text{C}_{42}\text{H}_{27}\text{F}_{2}\text{IrN}_{5} [\text{M} – \text{PF}_{6}]^{+}: 832.1860; \text{found: 832.1888.}\]

### 3.3 Results and discussions

The photophysical properties of 1 and 2 were first examined. As shown in Figure 3.1, under the excitation at 360 nm, 1 was weakly emissive. In comparison, 2 exhibited a stronger luminescence peak at about 541 nm. This further indicated that the reduction of 1 into 2 resulted from H$_2$S or hypoxia could produce a “switch on” luminescence signal. Absorbance spectra of 1 and 2 were also recorded (Figure 3.2) and other photophysical properties of these complexes were investigated as shown in Table 3.1.

![Luminescence spectra of complexes 1 and 2 in EtOH.](image)

**Figure 3.1.** Luminescence spectra of complexes 1 and 2 in EtOH. $\lambda_{\text{exc}}$ was set at 360 nm.
Figure 3.2. Absorbance spectra of the complexes (5 µM) in ACN: (a) complex 1; (b) complex 2.

Table 3.1. Photophysical profiles of the complexes.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Quantum yield</th>
<th>(\lambda_{\text{emi}} / \text{nm} )</th>
<th>Lifetime / (\mu\text{s} )</th>
<th>UV-Vis absorption (\lambda_{\text{abs}} / \text{nm} (\epsilon / \text{dm}^3 \text{mol}^{-1} \text{cm}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N.A.</td>
<td>541 (very weak)</td>
<td>N.A.</td>
<td>215 ((8.03 \times 10^4), 238 (8.01 \times 10^4), 262 (8.98 \times 10^4), 325 (4.32 \times 10^4), 417 (1.26 \times 10^4))</td>
</tr>
<tr>
<td>2</td>
<td>0.0124</td>
<td>541</td>
<td>4.389</td>
<td>213 ((4.64 \times 10^4), 259 (4.77 \times 10^4), 329 (1.92 \times 10^4), 421 (1.61 \times 10^4))</td>
</tr>
</tbody>
</table>

The feasibility of 1 for the detection of \(\text{H}_2\text{S}\) was investigated in EtOH. As shown in Figure 3.3, the probe exhibited a significant luminescence enhancement in the presence of \(\text{H}_2\text{S}\), allowing visual detection under UV illumination (Figure
3.3b). A time-course experiment was further performed to monitor its luminescence response behavior with the addition of H₂S (200 µM) in EtOH. As shown in Figure 3.4, the luminescence intensity of the solution enhanced with incubation time, achieving a plateau at 1.5 h under 37 °C. An incubation time of 1.5 h was thus selected for the subsequent experiments. Luminescence response of 1 to H₂S was next examined within the concentrations range of 10 to 300 µM. The results show that the luminescence of the probe increase with the increasing concentration of H₂S, exhibiting a ca. 38-fold luminescence enhancement at 300 µM. The linear relationship was established between 10 to 170 µM H₂S, and the limit of detection was estimated to 4.35 µM based on a formula LOD = 3σ/s, where σ represented the standard deviation of five blank samples and s represented the slope of the calibration curve.

**Figure 3.3.** (a) Luminescence spectra of complex 1 (10 µM) in EtOH with/without the addition of H₂S (200 µM). λ_{exc} was set at 360 nm. (b) Pictures of only complex 1 (left) and 1 with H₂S (200 µM, right) in EtOH under UV illumination.
Figure 3.4. (a) Time course experiment of complex 1 (10 µM) within H$_2$S (200 µM) in EtOH under the incubation at a temperature of 37 °C. $\lambda_{\text{exc}}$ was set at 360 nm. Luminescence emission from 470 and 730 nm was collected at time points of 0, 20, 40, 60, 80, 100 min in the presence of H$_2$S (200 µM). (b) Time-dependent luminescence at 541 nm of complex 1 (10 µM) in the presence of H$_2$S (200 µM), $\lambda_{\text{exc}}$ was set at 360 nm.

Figure 3.5. (a) Luminescence enhancement of complex 1 (10 µM) to different concentrations of H$_2$S (10–300 µM) in EtOH. (b) The relationship between luminescence at 541 nm of complex 1 (10 µM) and H$_2$S (10–300 µM) in EtOH. Inset figure was the linear response to H$_2$S from 10 to 170 µM, $\lambda_{\text{exc}}$ was set at 360 nm.
The selectivity profile of 1 was also investigated by using a range of analytes including biological thiols (GSH, Cys, Hey, GSSG, CSSC), inorganic sulfur species (SO$_4^{2−}$, SO$_3^{2−}$, HSO$_3^−$, SCN$^−$, S$_2$O$_4^{2−}$), anions (HCO$_3^−$, F$^−$, Cl$^−$, Br$^−$, HPO$_4^{2−}$, H$_2$PO$_4^−$, CHCOO$^−$, NO$_2^−$, N$_3^−$), and organic carbon source glucose. The results show that the presence of potentially interfering biological thiols and inorganic sulfur species (200 µM) did not trigger a luminescence response to 1 (10 µM) in EtOH, and the addition of anions and glucose (200 µM) also did not elicit an obvious effect on the luminescence intensity (Figure 3.6). In comparison, H$_2$S (200 µM) triggered a significant luminescence enhancement to 1 (10 µM) (Figure 3.6). Taken together, these data indicated that 1 is a highly selective luminescent probe for H$_2$S over other species.

Figure 3.6. Luminescence changes $\lambda_{em} = 541$ nm of 10 µM complex 1 in the presence of various potential interfering species (200 µM). The luminescence was
collected at 1.5 h under 37 °C incubation after the addition of interfering species, $\lambda_{\text{exc}}$ was set at 360 nm. The inset figure was luminescence spectra of complex 1 (10 µM) with the addition of interfering species.

To get insight into the sensing mechanism of the probe, we further study its detection behavior using $^1$H NMR and HRMS. As demonstrated in Figure 3.7, the presence of H$_2$S results in a characteristic singlet of the H6 proton of phen ligand for 1 upshift from 9.01 to 7.73 ppm. At the same time, several proton signals located between 8.00 and 8.85 ppm of 1 downshifted, suggesting that the strong electron-withdrawing nitro group was reduced to an electron-donating functional group. This result was further supported by the HRMS experiment, in which the mixture of 1 and H$_2$S exhibited the main peak centered at 832.1869, the same as the peak of its reduction product 2 (Figure 3.8).
Figure 3.7. The aromatic region of $^1$H NMR spectra of complex 1 within (a) or without (b) H$_2$S in MeOH-$d_4$/D$_2$O (9:1, v/v).

Figure 3.8. MALDI-TOF-HRMS of the product after complex 1 treated with H$_2$S in EtOH.

Before examining the feasibility of 1 to image H$_2$S in living cells, its stability and cytotoxicity were measured. $^1$H NMR spectroscopy was used to evaluate the stability of 1 (5 mM) at a time course of 7 days. The results show that the probe remained the same in a solution of 90% DMSO-$d_6$/10% D$_2$O during this time course (Figure 3.9). Its cytotoxicity towards LO2 cells and HeLa cells was determined by MTT assay. The probe exhibited dose-dependent inhibitions to LO2 cells and HeLa and their corresponding IC$_{50}$ values were 59.7 and 30.3 µM,
respectively. These indicate that 1 would not elicit significant cell toxicity using the required concentration (20 µM) in the imaging experiments (Figure 3.10). The water solubility of the probe was also determined by UV/Vis spectroscopy according to a previous report. The result shows that 1 has an acceptable solubility of 50.02 mg/L in H₂O (Figure 3.11). Taken together, this probe was a good biocompatible probe for H₂S, which was suitable for further imaging experiments.

**Figure 3.9.** The partial ¹H NMR spectra of complex 1 (5 mM) in DMSO-d₆/D₂O (9/1) mixture at 298 K over 7 days.
Figure 3.10. The cytotoxicity of the probe on HeLa and LO2 cells as determined by an MTT assay.

Figure 3.11. The solubility of the probe as determined by UV/Vis absorbance at 280 nm.

To investigate the feasibility of 1 for the detection of exogenous H₂S in living cells, confocal imaging was performed. In this work, Na₂S was used as the exogenous source of H₂S. Prior to the treatment with different concentrations of H₂S (50−400 µM), HeLa cells were pre-incubated with 1 (20 µM) for 1 h at 37 °C. Upon the incubation of the cells with increasing concentration of H₂S for 1 h, the
luminescence of the HeLa cells was recorded. The results show an increased luminescence intensity with an increasing concentration of H$_2$S (Figure 3.12). These data indicated that 1 was capable of imaging exogenous H$_2$S in living cells. Encouraged by these results, 1 was further applied to detect endogenous H$_2$S. Cys and GSH were previously reported to produce endogenous H$_2$S in living system.$^{64-66}$ HeLa cells were thus pre-incubated with Cys or GSH to produce endogenous H$_2$S for 1 h prior to the treatment with the probe (20 µM) for 1 h. As shown in Figure 3.13, the luminescence of the cells increased with an increasing concentration of Cys or GSH (30−100 µM). Taken together, 1 is capable of imaging not only the exogenous H$_2$S, but also the endogenous generated H$_2$S in living cells.

![Figure 3.12](image.png)

**Figure 3.12.** Luminescence responses of HeLa cells pre-treated with 20 µM complex 1 to different concentrations of H$_2$S (50−400 µM). HeLa cells were pre-incubated with 20 µM complex 1 for 1 h, then subjected to the treatment of vehicle control or H$_2$S (50−400 µM) for 1 h at 37 °C.
Figure 3.13. Confocal luminescence microscopy imaging of endogenous H$_2$S stimulated by Cys or GSH. HeLa cells were pre-incubated with Cys or GSH for 1 h before the treatment with 20 µM complex 1 for 1 h.

The detection of H$_2$S in living organisms was also investigated using zebrafishes, due to their rapid growth rate, close evolutionary relationship to humans and transport morphology.$^{67-69}$ Zebrafish embryos were first pre-treated with 1 (20 µM) at 72 hpf for 1 h, followed by the replacement with fresh medium or medium containing different concentrations of H$_2$S (200–800 µM). These embryos were then imaged with confocal microscopy at 74 hpf. As shown in Figure 3.14, only weak luminescence signal was detected in the embryos in the absence of H$_2$S, while considerable luminescence responses were observed with the addition of H$_2$S (200–800 µM). During the experiments, no observable toxicity of 1 to the embryos was found. These suggested that 1 was biocompatible.

<table>
<thead>
<tr>
<th>Complex 1</th>
<th>Cys</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 µM</td>
<td>100 µM</td>
</tr>
<tr>
<td></td>
<td>30 µM</td>
<td>100 µM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Complex 1</th>
<th>Cys</th>
<th>GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 µM</td>
<td>100 µM</td>
</tr>
<tr>
<td></td>
<td>30 µM</td>
<td>100 µM</td>
</tr>
</tbody>
</table>
to the living system and is a suitable *in vivo* probe for studying H$_2$S in a living organism.

**Figure 3.14.** Luminescence microscopy imaging of H$_2$S in zebrafish. Zebrafish embryos at 72 hpf were pre-incubated with complex 1 (20 µM) for 1 h. The embryos were then incubated with (A) fresh medium; (B) medium containing 200 µM H$_2$S; (C) medium containing 400 µM H$_2$S; (D) medium containing 800 µM H$_2$S.

The level of reductive enzymes has been reported to be elevated in the hypoxic cells or organisms, such as nitroreductase.$^{70, 71}$ Therefore, we also reasoned that 1 may serve as an oxygen deficiency indicator by responding to the elevated enzymatic activity of nitroreductase in hypoxic cells. In this work, hypoxic conditions of cells were generated by an AnaeroPack (Mitsubishi Gas Corp.) Complex 1 (20 µM) was added to the hypoxic HeLa cells (1% O$_2$, 5% CO$_2$)
or normoxic cells in a CO₂ incubator (20% O₂, 5% CO₂) and incubated for 12 h. Encouragingly, strong luminescence was observed in the hypoxic HeLa cells, while HeLa cells under normoxic conditions only showed weak luminescence (Figure 3.15). The concentration of 1 was also investigated in hypoxic HeLa cells, in which the luminescence of the cells under hypoxia enhanced with an increasing concentration of 1 (2–20 µM) (Figure 3.16). Additionally, the cells under hypoxia displayed an increased luminescence with incubation time (Figure 3.17). Taken together, 1 is capable of visualizing hypoxic living cells.

**Figure 3.15.** Luminescence images of hypoxic HeLa cells within 20 µM complex 1. Normoxic conditions were generated in a CO₂ incubator (20% O₂, 5% CO₂), and hypoxic conditions were produced in an AnaeroPack (1% O₂, 5% CO₂). HeLa cells were incubated under normoxic conditions or hypoxic conditions for 12 h with the addition of complex 1 (20 µM).
Figure 3.16. Luminescence images of hypoxic HeLa cells within indicated concentrations of complex 1. Complex 1 was incubated with hypoxic HeLa cells for 12 h.

Figure 3.17. Luminescence images of hypoxic HeLa cells which were incubated with complex 1 (6 µM) with indicated incubation time (3–12 h).

To confirm whether the luminescence of hypoxic cells was attributed to an
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enzyme-catalyzed reduction reaction, the enzyme activity in hypoxic cells was determined by a commercial nitroreductase (NTR) ELISA kit. As shown in Figure 3.18, the hypoxic cells displayed an increasing activity of NTR within increasing incubation time. To gain more insight into the sensing mechanism of 1 in hypoxic cells, the luminescence intensity of 1 was investigated in the presence of NTR. The luminescence enhancement of 1 (20 µM) was observed after incubating with NADH (500 µM) and NTR (5 µg/mL) at 37 °C for 1 hour, while 1 did not display luminescence response toward NADH (Figure 3.19), indicating that the luminescence did not arise from the direct interaction between 1 and NADH. Collectively, these data indicated that this probe can easily enter the living cells, and “light-up” the cells under hypoxic conditions, presumably due to its luminescence response to the increased reduction activity of NTR under hypoxia.

The ability of 1 to study the in vivo hypoxia was also evaluated in a zebrafish model. Cobalt(II) chloride (CoCl₂) is widely used to stimulate hypoxia in living systems.⁷²-⁷⁴ Therefore, we employed CoCl₂ to build a hypoxic zebrafish model. 3-day-old zebrafish embryos were incubated with a vehicle or CoCl₂ (5 mM) with/without the addition of 1 (20 µM) for 24 h. As shown in Figures 3.20A and 3.20C, no obvious luminescence was observed in the embryos in the absence of 1. However, in the presence of 1, the embryos treating with CoCl₂ displayed a much stronger luminescence when compared to the embryos not treating with CoCl₂ (Figures 3.20B and 3.20D). These data demonstrated the capability of 1 to visualize living organisms under hypoxia.
Figure 3.18. Changes of NTR activity with the increase of incubation time from 0 h to 12 h.

Figure 3.19. The relative luminescent intensity of complex 1 (20 µM), a mixture of complex 1 (20 µM) and β-nicotinamide adenine dinucleotide (NADH, 500 µM), or a mixture of complex 1 (20 µM), NADH (500 µM) and NTR (5 µg/mL). All were incubated in PBS buffer (pH 7.4) for 1 h at 37 °C. λ_{exc} = 360 nm.
Figure 3.20. Confocal luminescence microscopy imaging of hypoxic zebrafish with complex 1 (20 µM). The zebrafish embryos were incubated with (A) Fresh medium; (B) Medium containing complex 1 (20 µM); (C) Medium containing CoCl$_2$ (5 mM); (D) Medium containing complex 1 (20 µM) and CoCl$_2$ (5 mM).

3.4. Conclusion

In summary, a novel reaction-based luminescent Ir(III) complex was designed and synthesized for the detection of H$_2$S and hypoxia both \textit{in vitro} and \textit{in vivo}. The probe exhibits high selectivity and sensitivity for H$_2$S \textit{in vitro}, with a detection limit of 4.35 µM. The ability of 1 to detect H$_2$S in the living systems was verified using luminescence microscopy in living cells and zebrafish model. The results show that 1 could detect both the exogenous and endogenous H$_2$S in the living systems. Meanwhile, we have also demonstrated the capability of 1 to discriminate hypoxic cells from normoxic cells, presumably due to the elevated NTR activity in hypoxic conditions of the living cells. The ability of 1 to detect hypoxia was further demonstrated in a hypoxic zebrafish model. 1 shows great potential in the monitoring of both H$_2$S and hypoxia in living organisms.
3.5 Reference


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Chapter 4

Oridonin-conjugated Ir(III) complex for tracking NF-κB in living cells

4.1 Introduction

NF-κB, a transcription factor that exists in the cytoplasm of many cell types, plays a key role in cell proliferation, immune response, and inflammatory response. NF-κB belongs to the family of structurally related proteins and exists in the form of either heterodimers or homodimers.¹ Each family member contains a region terminated with an amino group, which are known as the Rel-homology domain, which consists of the nuclear localization signal as well as the dimerization, and the DNA-binding domains. To date, there are five NF-κB family proteins have been found in mammalian cells, including RelB, cRel, NF-κB (p52/p100), NF-κB1 (p50/p105), and RelA (also known as p65).² Among these protein types, RelA, RelB, and cRel are expressed in the form of transcriptionally active proteins, while NF-κB1 and NF-κB2 are first generated as precursor molecules and then further activated into transcriptionally active forms. While the p50 and p65 are known as the classic form of NF-κB dimer, other dimers containing Rel are also exist³.

There are various stimuli that are capable of activating NF-κB. Notable examples include T- and B-cell mitogens,⁴ proinflammatory cytokines,⁵ microbial products,⁶ as well as chemical and physical stresses.⁶ Without stimulation, NF-κB mostly remains in inactive form as an NF-κB/IκB complex in the cytoplasm.
However, the stimulated cells could initiate the phosphorylation process of the NF-κB/IκB complex by the IκB kinase complex (IKK), and then degrade the IκB proteins. As a result, the NF-κB is promoted to enter the nucleus and binds to its targeted DNA in the regulatory regions of genes, which mediate the gene expression.\(^7\)\(^-\)\(^9\)

Importantly, the binding sites for NF-κB are located in the promoter regions of most of the proinflammatory cytokines and immunoregulatory mediators, which could play a role in the regulation of acute inflammatory responses. As a result, the activation of NF-κB may be critical in the pathological of a number of dysfunctions including, multiple organ dysfunction, abnormal hormone responses, inflammation, developmental disorder, and even cancer.\(^10\)\(^,\)\(^11\) Considering the association of NF-κB with different diseases/disorders, visualization of intracellular NF-κB is of significant interest as it could provide information on both the stimulation responses and signaling pathway of NF-κB activation.

To date, a range of methods have been developed for the detection of NF-κB, including nanopores,\(^12\) cross-correlation spectroscopy,\(^13\) electrochemistry,\(^14\) fluorescence resonance energy transfer,\(^15\)\(^,\)\(^16\) as well as oligonucleotide-based platforms.\(^17\)\(^-\)\(^23\) Nevertheless, approaches for the intracellular tracking of NF-κB still remain scarce.\(^24\)\(^-\)\(^26\) Over the past years, applications of transition metal complexes, particularly Ir(III) complexes, have been extensively investigated as chemical and biological probes due to their attractive properties, including (i) tunable excitation and emission wavelengths, (ii) long emission lifetimes, (iii)
high quantum yields, as well as (iv) well-defined, octahedral geometry that could interact with biomolecules in a specific manner.\textsuperscript{27-34}

In this work, we integrated a natural product oridonin to an Ir(III) scaffold. This luminescent complex 1 was able to bind to the active NF-κB dimers (p50/p50 homodimer or p50/p65 heterodimer) and track the activities of intracellular NF-κB. Ir(III) complexes generally possess a long phosphorescence lifetime, which enables their emission to be readily distinguished from the highly-autofluorescence background in biological medias.

To date, studies on the conjugation of transition metal complexes to natural products are not extensively studied.\textsuperscript{35, 36} Significantly, the solubility of the Ir(III) moiety and oridonin are not the same in different solvents. Therefore, conjugation of oridonin to the N\textsuperscript{\textsc{n}} ligand of the Ir(III) scaffold in a direct manner would be very difficult. As a result, we conceived an alternative approach by dividing the N\textsuperscript{\textsc{n}} ligand into three components and assembled the structures step-by-step with protecting groups. After preparing the oridonin-incorporated N\textsuperscript{\textsc{n}} ligand, the target complex could be obtained readily in a relatively higher yield.

\section*{4.2 Experimental section}

\subsection*{4.2.1 Chemicals and general experimental}

Chemicals, commercially available, were bought from Sigma Aldrich (St. Louis, MO) or Precious Metals Online (Australia) and used without further purification. Luminescence spectra and emission lifetime for samples were recorded on a PTI
TimeMaster C720 Spectrometer, HRMS experiment was completed at the Mass Spectroscopy Unit at Hong Kong Baptist University. NMR experiments were performed on a Bruker Avance 400 spectrometer ($^1$H at 400 MHz, $^{13}$C at $s101$ MHz). DMEM and FBS were bought from Gibco BRL (Gaithersburg, MD, USA). HeLa and HepG2 cell lines were bought from the American Type Culture Collection (Manassas, VA, USA), and were cultured in the mixture DMEM/FBS (9/1) in 5% CO$_2$ incubator at 37 °C. 35 mm dish with 20 mm well glass-bottomed dishes were purchased from MatTek Corp. (Ashland, MA). XTT assay kit was bought from Cayman Chemical (Ann Arbor, MI, USA). Control siRNA and NF-κB p50 siRNA were bought from Santa Cruz Biotechnology (Santa Cruz, Canada). Cytoplasmic protein and the nuclear extraction kit were purchased from Thermo Fisher Scientific. Luciferase reporter assay system was purchased from Promega.

4.2.2 Synthesis

**Detailed steps in the synthetic route of complex 1**

![Chemical diagram]

Compound 1a was prepared according to modified reported method. In a
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round-bottomed flask, p-toluidine (1 eq), 1,10-phenanthroline-5,6-dione (1 eq), 4-hydroxy-3-nitrobenzaldehyde (1 eq) and ammonium acetate (10 eq) were mixed into acetic acid. Then the mixture was stirred and heated to reflux overnight. The resulting mixture was then cooled to rt, and poured into ice H₂O, adjusted to pH 7 by NH₄OH. The solution was then extracted by DCM three times, the organic layer was then combined and dried over Na₂SO₄, and the volatiles removed under reduced pressure. Silica gel column chromatography was carried out to purify the residue using the eluent (DCM/MeOH, 10/1, v/v) to yield the compound 1a. Yield: 75%. ¹H NMR (400 MHz; CDCl₃-d): δ 9.20 (dd, J = 4.0, 1.6 Hz, 1H), 9.14 (dd, J = 8.0, 1.6 Hz, 1H), 9.07 (dd, J = 4.4, 1.6 Hz, 1H), 8.00 (dd, J = 6.8, 2.0 Hz, 2H), 7.77 (dd, J = 8.0, 4.4 Hz, 1H), 7.71 − 7.68 (m, 2H), 7.52 (d, J = 8.8, 2.0 Hz, 1H), 7.46 − 7.40 (m, 4H), 7.32 (d, J = 8.4, 4.4 Hz, 1H); 3.92 (s, 3H); 2.57 (s, 3H).

Compound 1a (0.45 mmol) was dissolved into 6 mL MeOH, a solution of LiOH (1.13 mmol) in 2 mL H₂O was then added. The resulting suspension was heated to reflux for 5 h, and the volatiles were evaporated, followed by the addition of 30 mL H₂O. The solution was neutralized with con. HCl to form a white solid (compound 1b). Compound 1b was used without further purification because of its poor solubility.

In 2 mL freshly distilled SOCl₂, compound 1b (0.058 mmol) was slowly added over 5 min. The suspension was then heated to reflux for 3 h under a nitrogen atmosphere, changing into a green suspension. SOCl₂ was then evaporated under in vacuo to give the desired compound 1c. The product was immediately put into
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the next step without further purification and characterization.

Intermediate 1c was dissolved into 10 mL DCM, Et$_3$N (0.116 mmol), and oridonin (0.085 mmol) were added. The resulting solution was then stirred overnight at rt. The volatiles was evaporated to give the crude product *in vacuo*. Silica gel column chromatography was carried out to purify the residue using the eluent (MeOH/DCM, 1/10) to obtain compound 1d with a yield of 60%.

Complex 1 was prepared according to a modified reported method.$^{38}$ The Ir(III) dimer [Ir(ppy)$_2$Cl]$_2$ (0.0285 mmol) and compound 1d (0.063 mmol) were dissolved into the mixed solvent of 5 mL DCM and 5 mL MeOH. The resulting solution was stirred at rt overnight, followed by the addition of excess solid NH$_4$PF$_6$, the resulting solution was then stirred for further 30 min at rt. Then the solution was washed by H$_2$O, and the volatiles were removed *in vacuo* and silica gel column chromatography was performed to purify the residue using the eluent (MeOH/DCM, 30/1) to afford complex 1 as a brown solid with a yield of 64%.

Overall yield: 28.8%. $^1$H NMR (400 MHz, CDCl$_3$-d) $\delta$ 9.33 – 9.29 (d, $J$ = 9.2, 1H), 8.25 (d, $J$ = 5.0 Hz, 1H), 8.16 (d, $J$ = 5.1 Hz, 1H), 7.92 – 7.87 (m, 3H), 7.86 – 7.82 (m, 2H), 7.73 – 7.72 (m, 1H), 7.71 – 7.70 (m, 3H), 7.69 – 7.68 (m, 2H), 7.66 – 7.64 (m, 1H), 7.58 (dd, $J$ = 4.1, 2.3 Hz, 1H), 7.54 – 7.54 (m, 1H), 7.52 – 7.51 (m, 1H), 7.50 – 7.47 (m, 1H), 7.45 (d, $J$ = 5.2 Hz, 1H), 7.39 (d, $J$ = 11.2 Hz, 1H), 7.32 (d, $J$ = 5.2 Hz, 1H), 7.05 (dd, $J$ = 8.5, 7.2 Hz, 2H), 7.02 – 6.98 (m, 1H), 6.98 – 6.97 (m, 1H), 6.95 (d, $J$ = 1.4 Hz, 1H), 6.84 (t, $J$ = 6.0 Hz, 1H), 6.37 (d, $J$ = 7.5 Hz, 2H), 6.18 (s, 1H), 6.09 (d, $J$ = 1.8 Hz, 1H), 5.51 (s, 1H), 4.34 (d, $J$ = 10.1 Hz,
1H), 4.07 (d, $J = 10.3$ Hz, 1H), 4.00 (d, $J = 3.8$ Hz, 1H), 3.76 – 7.64 (m, 1H), 3.52 (dd, $J = 11.2$, 5.5 Hz, 1H), 3.27 (d, $J = 10.0$ Hz, 1H), 3.01 – 2.88 (m, 1H), 2.70 – 2.60 (m, 2H), 2.55 (s, 3H), 2.40 – 2.16 (m, 3H), 2.00 (dd, $J = 13.0$, 6.0 Hz, 3H), 1.11 (s, 3H), 1.09 (s, 3H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 168.20, 164.64, 149.81, 149.37, 149.02, 148.40, 145.13, 143.94, 143.54, 138.26, 138.18, 133.75, 133.08, 132.34, 132.11, 131.89, 131.17, 131.07, 130.80, 129.90, 129.53, 128.56, 127.62, 126.79, 126.72, 126.38, 124.95, 124.84, 123.97, 123.18, 123.00, 122.90, 119.80, 119.60, 96.28, 74.59, 73.48, 63.69, 62.28, 59.75, 54.95, 46.97, 46.12, 41.62, 41.52, 38.79, 33.86, 32.77, 30.73, 30.11, 29.45, 21.85, 21.75, 20.06, 8.78. HRMS: Calcd. for C$_{69}$H$_{62}$Ir$_6$N$_6$O$_7$ [M–PF$_6$]$^+$: 1277.4153. Found: 1277.4127.

(a)

(b)
4.2.3 Cell viability assay determined by XTT

In a 96-well plate, cells were seeded with the density of 5000 cells per well, followed by the incubation of 12 h at 37 °C. Various concentrations of complex 1 (0.01–100 µM) were added into each well, followed by the incubation in a
humidified incubator for three days at 37 °C. 50 µL of the prepared XTT mixture was added for 4 h. 100 µL of DMSO was used to replace the medium, followed by the incubation for 10 min at rt with shaking. A SpectraMax M5 microplate reader was used to measure color intensity at 450 nm.

### 4.2.4 Luciferase reporter assay

A luciferase reporter assay system was applied to determine the inhibition of NF-κB activity according to a previous report. 4 µg pRL-TK plasmid, 4 µg pNF-κB-luc and 6 µL TurboFect reagent were first mixed in DMEM, which was then added dropwise into the wells containing HeLa cells for 32 h at rt. Different concentrations of complex 1 were added into transient transfected HeLa cells for 4 h. The cells were treated with or without 25 ng/mL TNF-α for stimulation before measurement. A SpectraMax M5 microplate reader was used to measure before luciferase activity was integrated over a 0.75 second period. The activity of Renilla luciferase was used as a standard.

### 4.2.5 p50 knockdown assay

In 6-well plates, HeLa cells were seeded at 75% confluence in DMEM for 24 h. NF-κB p50 siRNA and Lipo2000 reagent were gently mixed, followed by the incubation for 20 min at rt. The medium was then replaced by 0.5 mL fresh medium. After the addition of 500 µL mixed Lipo2000/siRNA solution into each well, the plates were incubated in a CO₂ incubator for 48 h at 37 °C.
4.2.6 Cell imaging\textsuperscript{41}

In a 5% CO\textsubscript{2} incubator, HeLa cells were cultured for two days in the mixture of 90% DMEM and 10% FBS with penicillin/streptomycin at 37 °C. In a 35 mm glass-bottomed dish with 20 mm wells, HeLa cells were seeded for 12 h. Complex I was added into cells for further 4 h, while Hoechst for 10 min. These cells were then washed by PBS three times. The images of the cells were taken by a Leica TCS SP8 confocal microscope. $\lambda_{\text{exc}}$ was set at 405 nm.

4.2.7 Western blotting\textsuperscript{42}

TNF-α was added into HeLa cells with a density of $2 \times 10^6$ cells per well for the indicated incubation time. The cells were then washed by cold PBS. A cytoplasmic protein or nuclear extraction kit was then used to collect protein samples. SDS-PAGE gels electrophoresis was employed to separate 20 µg protein samples. The separated samples were then transferred on the polyvinylidene difluoride (PVDF) membrane, blocking solution was subsequently added and incubated for 1 h at rt. Primary antibodies were used to treat the membrane overnight at 4 °C, followed by the incubation with secondary antibodies. Finally, A ECL Western Blotting Detection Reagent was used to visualize protein bands.

4.3 Results and discussions

The synthesis of complex I was outlined in Figure 4.1. When designing complex
three aspects have to be taken into consideration, including the synthesis of the oridonin-conjugated N^N ligand, the luminescence properties of the parent Ir(III) complex after integration to oridonin, and the NF-κB recognition ability of oridonin after integrating to the Ir(III) complex. The previous studies suggested that the functionalization of the 14-OH group on oridonin would not affect its biological activity against different cancer cells. In addition, several groups have recently reported the selective acylation of the 14-OH group on oridonin. Therefore, the 14-OH group of oridonin is a preferred location to conjugate the N^N ligand. In this work, (4-(1-(p-tolyl)-1H-imidazo[4,5-f][1,10]phenanthroline-2-yl)benzoic acid) (1b) was also selected as the parent N^N ligand for oridonin conjugation because of its promising luminescence properties as reported.

The synthesis of complex 1 starts from complex 1a, which was synthesized from p-toluidine, methyl 4-formylbenzoate, and 1,10-phenanthroline-5,6-dione in a multicomponent Radziszewski reaction with 75% yield, which is similar to the procedure in Chapter 2. After that, 1a was refluxed in lithium hydroxide in MeOH/H_2O to be hydrolyzed. The reaction was completed in 5 h as shown in the thin layer chromatography (TLC) analysis. Acidification with con. HCl afforded 1b in HCl salt formation. Without further purification of 1b, the reaction was directly promoted to acylate the 14-OH group of oridonin because of its poor solubility in a range of organic solvents including ethyl acetate, DCM, and MeOH. Compound 1b was then converted into the acid chloride form 1c by the use of
thionyl chloride (SOCl₂). It was immediately employed to selectively acylate the oridonin. The acylation of the 14-OH group of oridonin was achieved in DCM. Column chromatography (DCM/MeOH) was employed to purify the crude product and yield compound 1d in a yield of 60%. The compound was then characterized using ¹H NMR, ¹³C NMR, and HRMS.

With oridonin-containing ligand 1d in hand, complex 1 was prepared by the reaction between Ir(III) dimer [Ir(ppy)₂Cl]₂ and ligand 1d in a mixture of DCM/MeOH (1:1) at rt overnight, followed by the addition of NH₄PF₆. The resulting mixture was further stirred for 0.5 h. The product was washed several times with H₂O. The organic layer was subsequently reduced in vacuo to obtain crude product was purified using preparative TLC. 1 was then characterized by ¹H NMR, ¹³C NMR, and HRMS spectroscopy.

**Figure 4.1.** Synthesis of complex 1. Reagents and conditions: a) ammonium acetate, HOAc, reflux, overnight, 75%; b) LiOH•H₂O, MeOH, H₂O, reflux, 5 h, 90%; c) SOCl₂, reflux, 3 h; (d) oridonin, Et₃N, DCM, rt, overnight, 60%; e) Ir(III) dimer [Ir(ppy)₂Cl]₂, DCM/MeOH (1/1), rt, overnight, then NH₄PF₆, 64%.
The photophysical profiles of 1 were outlined in Table 4.1. When excited at 330 nm, 1 displays a maximum emission at 577 nm. 1 showed a large Stokes shift of approximately 277 nm which is substantially larger than that of the organic dyes (Figure 4.2). As shown in Table 4.1, complex 1 exhibits a moderate absorption at 381 nm, which was assigned to its metal-to-ligand charge-transfer (MLCT) state. Furthermore, 1 exhibits a long phosphorescence lifetime of ca. 4.609 µs, which is significantly larger than the organic chemosensors that only show lifetimes at the range of nanoseconds. Remarkably, as demonstrated by UV/Vis spectroscopy, 1 was stable in a solution containing 20% 20 mM Tris-HCl buffer with 20 mM NaCl (pH 7.5)/80% ACN at 298 K for 7 days (Figure 4.2).

Table 4.1. Photophysical profiles of complex 1.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Quantum yield</th>
<th>( \lambda_{\text{emi}} ) (nm)</th>
<th>Lifetime (µs)</th>
<th>UV-Vis absorbance ( \lambda_{\text{abs}} / \text{ nm (} \varepsilon / \text{ dm}^3 \text{ mol}^{-1} \text{ cm}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.050</td>
<td>577</td>
<td>4.609</td>
<td>259 (0.83 ( \times 10^5 )), 282 (0.86 ( \times 10^5 )), 381 (1.40 ( \times 10^4 ))</td>
</tr>
</tbody>
</table>

(a)
Figure 4.2. UV/Vis absorbance of complex 1. (a) UV/Vis absorbance of 5 µM complex 1 in ACN; (b) UV/Vis absorbance of 10 µM complex 1 in ACN/20 mM Tris-HCl buffer (pH 7.5) (8/2) with 20 mM NaCl for seven days at rt.

Additionally, the long phosphorescence lifetime of 1 enables its emission to be differentiated from the high autofluorescence background using TRES. In this part, an organic dye named thioflavin S was employed as a fluorescent interference to 1. When the time gate was set to before the decay time of thioflavin S, a strong fluorescence peak centered at 440 nm was found and the extending tail of this peak perturbed the emission of 1 (Figure 4.3a). In contrast, if
the delay time was preset to be longer than thioflavin S, the emission peak of thioflavin S was eliminated and the emission of 1 become more evident (Figure 4.3b).

![Time-resolved luminescence spectra of complex 1 with the addition of thioflavin S.](image)

**Figure 4.3.** Time-resolved luminescence spectra of complex 1 with the addition of thioflavin S. (a) Time gate was set at before the delay of thioflavin S. (b) Time gate was after the delay of thioflavin S.

We next investigated the capability of 1 as a tracking probe for NF-κB in the living system (Figure 4.4). We first incubated the human cervical cancer cells HeLa with 1 at 37 °C for 4 h, followed by the stimulation of TNF-α before visualizing with confocal microscopy. After stimulating with TNF-α, the translocation of NF-κB from the cytoplasm to nucleus was visualized by 1 in HeLa cells. Upon stimulation with TNF-α for 10 min, an obvious yellow emission was observed in the cytoplasm, suggesting that the active NF-κB was mainly located in the cytoplasm initially (Figure 4.5a). After stimulation with TNF-α for 20 min, luminescence intensity was transferred to the nuclear region and was
overlapped with the luminescence of the nuclear staining agent Hoechst 33342 (Figure 4.5b). The results demonstrate that 1 is capable of tracking the active NF-κB in the living cells. To verify the binding of 1 to NF-κB and the formation of 1-NF-κB, Western blotting was performed to quantitatively measure the nuclear translocation of the active NF-κB. After stimulating with TNF-α, the images show that the NF-κB level substantially decreased in the cytoplasm decreased (Figure 4.5c) but significantly increased in the nucleus (Figure 4.5d). These indicate the induction of NF-κB translocation by TNF-α in HeLa cells and further demonstrate the ability of 1 to recognize and bind to NF-κB specifically without affecting the translocation process of NF-κB. Remarkably, 1 exhibits high chemical stability and photostability, with stable emission intensity after incubating with cell culture medium or cells for 4 h. These suggest that 1 could track NF-κB intracellularly over long time e.g. a full cell cycle, in contrast to most of the reported fluorescence organic probes with low photostability.\textsuperscript{14, 19}

\textbf{Figure 4.4.} Schematic diagram of Ir(III) complex 1 for tracking active NF-κB in...
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Figure 4.5. Tracking TNF-α induced NF-κB by using complex 1 in living cells. (a) HeLa cells were treated by 10 µM 1 for 4 h, followed by being stimulated by 25 ng/mL TNF-α for 10 min. (b) HeLa cells were treated by 10 µM 1 for 4 h, followed by being stimulated by 25 ng/mL TNF-α for 20 min. Hoechst 33342 was used to stain the cells before imaging. (c) The levels of cytoplasmic NF-κB in HeLa cells with or without 25 ng/mL TNF-α stimulation. (d) The levels of nuclear NF-κB in HeLa cells with or without 25 ng/mL TNF-α stimulation.

The binding ability of 1 to NF-κB was verified by performing the competition experiment with isolated oridonin. Oridonin, a natural product that capable of binding to the p50 subunit of NF-κB, could disrupt the interaction of NF-κB with dsDNA. HeLa cells were preincubated with oridonin for 1 h and then incubated with 1 for 4 h. After that, 25 ng/mL TNF-α was added to stimulate the
NF-κB for 20 min. The luminescence intensity in the nuclear regions of HeLa cells decreases substantially after pretreating with oridonin (Figure 4.6). These data demonstrate that oridonin could block the interaction between NF-κB and 1 in HeLa cells, presumably via competitive inhibition to the p50 subunit.

**Figure 4.6.** Competition imaging experiment by an NF-κB inhibitor oridonin which competes with complex 1 for binding to NF-κB in HeLa cells. HeLa cells were incubated with 10 µM complex 1 and indicated concentrations of oridonin for 4 h, followed by 25 ng/mL TNF-α stimulation for 20 min. Hoechst 33342 was used to stain the cells before imaging. Merge, a merged image.

We next confirmed the ability of 1 to target the p50 subunit in a specific manner. A p50 subunit of NF-κB knockdown experiment was employed, HeLa cells were first transfected by NF-κB p50 siRNA. Western blot analysis reveals
that the expression of p50 was reduced significantly in the transfected cells (Figure 4.7). In normal HeLa cells, the luminescence of 1 mainly accumulated in the cytoplasmic region with weak luminescence located in the nucleus. In contrast, in the transfected cells, only weak luminescence located in both cytoplasmic and nuclear regions (Figure 4.8), indicating that 1 was highly selective to the intracellular p50 subunit of NF-κB.

**Figure 4.7.** The results of a p50 knockdown assay in HeLa cells. Control siRNA or p50 siRNA were incubated with HeLa cells for 48 h. Cell extracts were prepared and protein samples were collected to perform Western blotting analysis.
Figure 4.8. Luminescence imaging of HeLa cells after a p50 knockdown experiment. Normal HeLa cells and p50 knockdown HeLa cells were incubated with or without 10 µM complex 1 for 4 h, followed by 25 ng/mL TNF-α stimulation for 20 min. Hoechst 33342 was used to stain the cells before imaging. Merge, a merged image.

Furthermore, to investigate the effect of 1 on the activity of NF-κB, a luciferase reporter assay was also performed (Figure 4.9a). The result shows that 1 only imposed negligible effect on the transcriptional activity of NF-κB with an IC\(_{50}\) > 100 µM in HeLa cells. Moreover, (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl) -2\(H\)-tetrazolium-5-carboxanilide) (XTT) assay was further employed to study the cytotoxicity of 1 (Figure 4.9b), with a IC\(_{50}\) of > 100 µM against HeLa cells after 72 h exposure. The IC\(_{50}\) was 10 times higher than the concentration used for NF-κB tracking in HeLa cells (10
µM). These data demonstrate the ability of 1 for tracking NF-κB in living cells without affecting their normal proliferation and other functions.

**Figure 4.9.** NF-κB activity assay of complex 1 and cytotoxicity effect of complex 1 towards HeLa cells. (a) HeLa cells were transiently transfected by pNF-κB-luc and pRL-TK plasmid for 32 h. Different concentrations of complex 1 (0.1–100 µM) were then added into the transfected HeLa cells for 4 h. 25 ng/mL TNF-α was used to stimulate the cells before imaging. A luciferase reporter assay was used to determine the NF-κB activity. (b) Cytotoxicity effect of complex 1 towards HeLa cells was measured by an XTT assay.

Basically, fluorophores can be classified into three sub-types, including “always-on”, “switch-on” or “switch-off”, \(^{47,48}\) depends on their type of interaction in the presence of the analytes. Among the three sub-types, “switch-on” and “always on” types are less suspected to the interference of the background noise and could lower the target-to-background ratio. However, the results of “turn-off”
mode of detection could be easily influenced by the biological interferences via different energy transfer mechanisms and may susceptible to false-positive.\textsuperscript{47, 49} As a result, “switch-on” and “always on” modes of detection are more preferable for imaging. In this work, a natural product oridonin was integrated to an Ir(III) complex to serve as a permanent luminescence probe to visualize NF-κB.

For the preparation of 1, we conceived an alternative approach by dividing the N^N ligand into three compartments and assembled the structures step-by-step with protecting groups. With the assistance of the protecting group, we successfully obtained the targeted N^N ligand in a good yield, which was then employed to couple with the Ir(III) moiety. As the solubility of Ir(III) moiety and oridonin are not the same in different solvents, this approach is superior to directly integrate the oridonin-conjugated N^N ligand to Ir(III) complex. Significantly, the combination of Ir(III) complex with oridonin allows 1 to recognize NF-κB with retaining its favorable photophysical properties.

Additionally, the yellow emission of 1 in the confocal images could be easily discriminated from the signals of Hoechst dye or the background, allowing efficient tracking of the translocation processes of NF-κB. We also confirmed that 1 could selectively bind to the p50 subunit of NF-κB over other biomolecules inside living cells with complicated environments, as shown in the knockdown assay. Meanwhile, we also demonstrated the ability of 1 to target NF-κB in living cells, as evidenced by the significantly lowered luminescence intensity of 1 in the presence of oridonin. Significantly, 1 was capable of visualizing the TNF-α
induced translocation of NF-κB without posing any effect on the translocation process of TNF-α.

Remarkably, 1 shows high photostability and chemical stability in cells and cell culture media. Luciferase and XTT assays also confirmed that 1 would not substantially influence the normal functions of NF-κB or cell viability at the concentration employed in the tracking of NF-κB. These results demonstrate the ability of 1 to monitor NF-κB in the living system over a relatively long period of time, without posing the toxic effect to the cellular system. Finally, we have demonstrated the long emission lifetime of 1 and its applicability to overcome the potential issues such as the autofluorescence interferences. To our knowledge, this is the first report that a metal-based compound was used for the tracking of the protogenetic NF-κB directly in the living system, allowing us to further explore the pathways or stimulation responses involving the activation of NF-κB. Compared with the previously reported probes that indirectly target the modified tag of non-protogenetic NF-κB,50-55 our approach shows high potential for studying the endogenous events.

4.4 Conclusion

In conclusion, Ir(III) complex 1, a complex integrating the natural product oridonin to an Ir(III) scaffold, was developed as a tracking agent of the translocation activity of the active NF-κB intracellularly. Further studies will be focused on the binding kinetics and mechanism of 1, and investigate its
applicability to study the NF-κB-associated activity in the living system. The
promising properties of 1 allow it to be a potential probe for understanding the
stimulation responses and signaling pathway involving the activation of NF-κB.
Finally, the practical synthetic strategies in this work may pave a way for the
discovery of more efficacious metal complexes as efficient probes for studying
other intracellular biological biomolecules.
4.5 Reference


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2006, **1109**, 14.
Chapter 5

Long-lived luminescence probe for detecting β-galactosidase in ovarian cancer cells

5.1 Introduction

β-Galactosidase (β-Gal) is an enzyme known as glycoside hydrolase, which is capable for the formation of monosaccharides by cleaving the glycosidic bond of β-galactosides. β-Gal is important for regulating gene expression due to their role in the development of Jacob-Monod's operon model. Furthermore, β-Gal is suggested to be a significant biomarker for primary ovarian cancer and cell senescence. In particular, β-gal is considered as a significant biomarker for ovarian cancer. Over the past years, a number of approaches have been developed for detecting β-gal, including histochemical methods, colorimetric assays, and immunostaining assays. Nevertheless, these methods generally limited by their drawbacks including complicated sample preparation, low sensitivity, and high cost.

Moreover, confocal imaging has emerged as a powerful approach to visualize biomolecules in the living systems. It shows advantages such as high compatibility with living cells, high sensitivity, and is thus considered as a minimally invasive detection mode. To date, there are several luminescent probes developed for the detection of β-gal in living systems. Nevertheless, traditional fluorescent probes are easily interfered by the highly autofluorescent
background of biological medias. Meanwhile, detection probes of β-gal in ovarian cancer cells remain scarce,\textsuperscript{28-31} while some examples only employed \textit{lacZ}-transfected cells with high expression of β-gal.\textsuperscript{15,16,19-27} Recently, Urano et al. synthesized a luminescence probe for the monitoring of β-gal activities in several ovarian cancer cell lines.\textsuperscript{28} However, the authors did not explore the imaging ability of the probe to discriminate ovarian carcinoma cells from normal cells.

Herein, a cyclometalated Ir(III) complex \textbf{1} was synthesized and developed as a long-lived phosphorescence probe for the activity of β-gal in living cells (Scheme 5.1). The photophysical properties of these octahedral Ir(III) complexes are highly associated with the sensing mechanism in this work, where alterations of the local environment or the substituting pattern on the phenanthroline scaffold may affect the emission of the probe.\textsuperscript{32-35} Therefore, the galactose moiety on \textbf{1} simultaneously acts as an emission quencher and an enzyme sensitive group. After hydrolyzing by β-gal, complex \textbf{1} is dealkylated to form complex \textbf{2}, which gives a yellow emission. To the best of our knowledge, \textbf{1} is the first Ir(III) complex-based probe for β-gal and is capable of distinguishing normal cells from ovarian cancer cells.
Scheme 5.1. Schematic diagram showing β-gal detection, and the chemical structures of 1 and 2.

5.2 Experimental section

5.2.1 Materials and general experimental

Materials and chemicals, commercially available, were purchased from J&K Chemical Ltd. (China), Sigma Aldrich (St. Louis, MO), Precious Metals Online (Australia), Gibco BRL (Gaithersburg, MD, USA) or New England Biolabs (Massachusetts, USA) and used without further purification. Cell lines, unless specified, were bought from the American Type Culture Collection (Manassas, VA, USA). Cell lines including OVCAR3 and SKOV3 were obtained from Dr. Wen-An Qiang as a generous gift. Luminescence spectra and emission lifetime for samples were recorded on a PTI TimeMaster C720 Spectrometer, HRMS was performed at the Mass Spectroscopy Unit at Hong Kong Baptist University. NMR experiments were performed on a Bruker Avance 400 spectrometer (13C at 101 MHz, 1H at 400.1 MHz).
5.2.2 Synthesis of complexes 1 and 2

**Compound S2**\(^{36,37}\): 900 mL commercial hypochlorite (CLOROX) was stirred in a round-bottomed flask at 18 \(^\circ\)C, tetrabutylammonium hydrogensulfate (0.06 mol) was added. Aqueous con. HCl was then added to the resulting solution to adjust pH to 8.2–8.3. A solution of compound S1 (0.015 mol) in 300 mL DCM was added, and 50% aqueous NaOH solution was used to maintain the pH of the mixture between 8.2 and 8.6. TLC was used to monitor the reaction. After the reaction, the organic layer was washed and separated by cold H\(_2\)O. The organic solution was dried over anhydrous Na\(_2\)SO\(_4\), and followed by the evaporation *in vacuo* to give solid. The residue was added into a mixture of chloroform-acetone (1/3). The product was obtained by filtration as a yellow powder in 92% yield. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.91 (d, \(J = 4.6\) Hz, 2H), 8.01 (d, \(J = 7.6\) Hz, 2H), 7.41 (dd, \(J = 7.6, 4.7\) Hz, 2H), 4.63 (s, 2H). MALDI-TOF-HRMS: Calcd. for C\(_{12}\)H\(_8\)N\(_2\)O: 196.0637. Found: 197.0700.

**Compound S3**\(^{37}\): Compound S2 (3.06 mmol) was gently added into a stirred 20 mL con. H\(_2\)SO\(_4\) under ice-water bath over about 40 min. The suspension was heated to 100 \(^\circ\)C for 1.0 h, along with the color change from yellow to dark brown. The resulting solution was cooled to rt, followed by adding 100 mL cold H\(_2\)O to dilute H\(_2\)SO\(_4\). 50% aqueous NaOH solution was slowly added into the solution to adjust pH to 7, while the temperature was kept strictly between 0 and −5 \(^\circ\)C by using a salt-ice bath. At 7 of pH, a very fine maroon powdery precipitate formed. The precipitate is filtered and dried own to obtain 500 mg (83%) of compound S3.
as a dark red solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.90 (s, 1H), 9.08 (dd, $J = 4.2, 1.7$ Hz, 1H), 8.83 (dd, $J = 4.2, 1.6$ Hz, 1H), 8.63 (dd, $J = 8.2, 1.7$ Hz, 1H), 8.24 (dd, $J = 8.1, 1.5$ Hz, 1H), 7.75 (dd, $J = 8.2, 4.3$ Hz, 1H), 7.60 (dd, $J = 8.1, 4.3$ Hz, 1H), 7.12 (s, 1H).

**Synthesis of S5 (1,10-phenanthroline-5-Gal)**\(^{38}\): Compound S3 (0.511 mmol), Cs\(_2\)CO\(_3\) (3.55 mmol) was added in 30 mL ACN and stirred at ambient temperature. 2,3,4,6-tetra-O-acetyl-alpha-D-galactopyranosyl bromide (301 mg, 0.7320 mmol) in ACN (30 mL) was added dropwise and stirred in the dark at ambient temperature for 12 h, followed by the filtration with a celite pad. After evaporating the filtrate, the residue was extracted with DCM three times, and the organic layers were then combined. The solution was then washed by brine, dried using Na\(_2\)SO\(_4\). The solution was removed under reduced pressure, to give the crude product. The residue was then purified by column chromatography (silica gel) using the eluent DCM/MeOH (9/1, v/v) to give a compound S4. Compound S4 was dissolved in 10 mL MeOH, and 100 µL of 4M NaOMe in MeOH was added. The resulting solution was stirred at ambient temperature for 2 h, and 1 mL of H\(_2\)O was then added to quench the reaction. The resulting solution was concentrated, and then purified using silica gel column chromatography with CH\(_2\)Cl\(_2\)/MeOH (9/1) as the eluent to afford 1,10-phenanthroline-5-Gal S5 (80 mg, 44%) as a yellow solid.

**Synthesis of complex 1**: 1 was synthesized based on modified previous reports.\(^{38, 39}\)

\(^{39}\) In brief, Ir(III) dimer [Ir\(_2\)(4-Fpq)\(_4\)]Cl\(_2\) (1 eq) (where ppy = 2-phenylpyridine)
and corresponding N^N ligand S5 (2.2 eq) ligands were dissolved into a mixed solvent of DCM:MeOH, the resulting mixture was stirred overnight. The reaction solution was treated according to previous work-up procedure. Yield: 60%, overall yield: 20.2%. \(^1\)H NMR (400 MHz, MeOD) \(\delta\) 9.01 (d, \(J = 8.6\) Hz, 1H), 8.56 (d, \(J = 8.4\) Hz, 1H), 8.26 (d, \(J = 4.9\) Hz, 1H), 8.09 (d, \(J = 4.9\) Hz, 1H), 8.03 (d, \(J = 8.2\) Hz, 2H), 7.85 – 7.81 (m, 1H), 7.81 – 7.75 (m, 2H), 7.71 (dd, \(J = 11.2, 4.1\) Hz, 2H), 7.35 (dd, \(J = 10.3, 5.4\) Hz, 2H), 6.98 (t, \(J = 7.6\) Hz, 2H), 6.83 (dd, \(J = 14.4, 8.2\) Hz, 4H), 6.35 – 6.27 (m, 2H), 5.31 (d, \(J = 7.8\) Hz, 1H), 4.82 (m, 1H), 4.75 (m, 1H), 4.73 (m, 1H), 3.89 (d, \(J = 9.8\) Hz, 1H), 3.63 (dd, \(J = 15.8, 6.8\) Hz, 3H), 3.55 – 3.45 (m, 1H), 3.45 – 3.31 (m, 2H). \(^{13}\)C NMR (101 MHz, MeOD) \(\delta\) 160.07, 152.15, 151.07, 148.68, 143.99, 140.08, 138.19, 137.61, 133.72, 131.45, 130.09, 126.15, 124.62, 122.99, 99.11, 76.48, 75.87, 73.62, 73.37, 69.95, 61.16. HRMS: Calcd. for C\(_{40}\)H\(_{34}\)F\(_{6}\)IrN\(_{4}\)O\(_{6}\)P [M–PF\(_{6}\)]\(^+\): 859.2104 Found: 859.2120.

**Synthesis of complex 2:** 2 was synthesized based on a previous report. Ir(III) dimer [Ir\(_2\)(4-Fpq)\(_4\)Cl\(_2\)] (1 eq) (where ppy = 2-phenylpyridine) and corresponding N^N ligand S3 (2.2 eq) ligands were dissolved into a mixed solvent of DCM:MeOH, the resulting solution was stirred overnight. The reaction solution was treated according to previous work-up procedure. \(^1\)H NMR (400 MHz, MeOD) \(\delta\) 8.84 (dd, \(J = 8.4, 1.3\) Hz, 1H), 8.26 (dd, \(J = 8.4, 1.1\) Hz, 1H), 8.18 (dd, \(J = 5.0, 1.4\) Hz, 1H), 8.00 (d, \(J = 8.2\) Hz, 2H), 7.88 (dd, \(J = 4.9, 1.1\) Hz, 1H), 7.70 (m, 5H), 7.52 (dd, \(J = 8.3, 5.0\) Hz, 1H), 7.34 (t, \(J = 5.6\) Hz, 2H), 7.16 (s, 1H), 6.94 (m, \(J = 5.7, 5.1, 2.5\) Hz, 2H), 6.81 (m, 4H), 6.29 (t, \(J = 7.9\) Hz, 2H). \(^{13}\)C NMR
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(101 MHz, MeOD) δ 149.97 (d, J = 5.8 Hz), 139.49 (s), 133.00 (d, J = 8.3 Hz), 131.47 (d, J = 5.0 Hz), 125.96 (d, J = 5.5 Hz), 124.32 (s), 123.63 (d, J = 4.5 Hz), 120.91 (d, J = 3.4 Hz), 49.45 (s), 49.24 (s), 49.02 (s), 48.81 (s), 48.60 (s). HRMS: Calcd. for C$_{40}$H$_{34}$F$_6$IrN$_4$O$_6$P [M–PF$_6$]$^+_{-}$: 679.1575 Found: 679.1545.

5.2.3 Stability experiment

The stability of complex 1 was determined by $^1$H NMR spectroscopy and UV absorbance. 5 mM complex 1 was kept in the mixture of 90% DMSO-$d_6$ and 10% D$_2$O (v/v = 9:1) at 298K for 72 h, $^1$H NMR spectra were recorded by $^1$H NMR spectroscopy each 24 h. On the other hand, complex 1 was also kept in the mixture of 80% ACN and 20% 20 mM Tris-HCl buffer (pH 7.4) containing 20 mM NaCl for 72 h at 298K. Absorption spectra were measured with Cary UV-100 Spectrophotometer each 24. For the photostability experiment, complex 1 with a final concentration of 5 µM and thioflavin T (ThT) were prepared in PBS (pH 7.0). Time-based luminescence spectra were measured over a course of 1800 s under the excitation of 355 nm on a PTI TimeMaster C720 Spectrometer. The emission of complex 1 and ThT was set at 586 nm and 427 nm, respectively.

5.2.4 β-Gal detection

Complex 1 was dissolved into DMSO as a stock solution with a concentration of 1 mM. Complex 1 was diluted into PBS buffer (2×, pH 7) with a final concentration of 2.5 µM, followed by the incubation with indicated concentrations of β-gal for
1.5 h at 37 °C. A PTI QM-4 spectrofluorometer was then used to record luminescence spectra of samples.

5.2.5 Time-resolved luminescence spectra measurement

Time-resolved luminescence spectra were collected on a time-correlated single photon counting (TCSPC) technique with the Horiba fluorescence spectrometer (FL3C-21). The excitation wavelength was set at 355 nm, while emission signals were collected between 400 and 700 nm with an interval of 10 nm. Cm460 and rhodamine B were used as fluorescent interferences. After they (0.1 µM) were added into the solution of 5 µM complex 1 in PBS buffer (2×, pH 7.0), respectively, the time-resolved luminescence spectra of the samples were acquired at a delay time of 0 ns and 333 ns, with or without the addition of β-gal (30 U/mL).

5.2.6 Cell viability assay

In 96-well plates, SKOV3 and HUVEC cells were seeded with a density of 5,000 cells per well for 12 h incubation. The indicated concentrations of 1 were added to the cells at different times (12–48 h), respectively. Then, 10 µL MTT reagent (5 mg/mL) was added and incubated for 4 h in the dark. 100 µL of DMSO was added to each well, and a SpectraMax M5 microplate reader was used to record the absorbance at 570 nm.
5.2.7 Luminescence cell imaging
Cells were first seeded for 12 h in a 35 mm glass-bottomed dish with 20 mm wells. Complex 1 was added into the cells with indicated concentrations for the indicated times. These cells were then washed by PBS three times. A Leica TCS SP8 confocal microscope was used to take images of complexes in cells. $\lambda_{\text{exc}}$ was set at 405 nm.

5.2.8 Confocal imaging with a $\beta$-gal inhibitor $D$-galactose
In a glass-bottomed dish, SKOV3 cells were first seeded in DMEM for 12 h, which is the 35 mm dish with 20 mm wells. A$\beta$-gal inhibitor $D$-galactose (10 mg/mL) was treated SKOV3 cells for 1 h. Then, SKOV3 cells were treated with 1 at 37 °C for 12 h in a CO$_2$ incubator. These cells were then washed with PBS three times, a Leica TCS SP8 confocal microscope was used to take images of complex 1 in SKOV3 cells. $\lambda_{\text{exc}}$ was set at 405 nm.

5.2.9 Determination of $\beta$-gal activity
A $\beta$-gal assay kit was performed to determine $\beta$-gal activity based on the manufacturer’s procedure. A lysis buffer was used to harvest Cells after the incubation of 24 h at 37 °C. 50 µL Test reagent and 50 µL cell lysate were added into 96 wells, followed by incubating at 37 °C for 3 h. 150 µL The reaction was then quenched using 150 µL Stop solution. A SpectraMax M5 microplate reader was applied to determine the absorbance of samples at 420 nm.
5.3 Results and discussion

The synthetic procedures for 1 and 2 are outlined in Figure 5.1. The synthesis of 1 started with reacting the commercially available S1 with aqueous HClO (pH 8.2–8.3) to generate S2.\(^{36,41}\) S2 was added slowly at a time course of 40 min while stirring with the con. H\(_2\)SO\(_4\) at 0 °C. The resulting suspension in yellow color was heated up in 100 °C for 1 h. The reaction solution was then diluted with cold H\(_2\)O, followed by neutralizing to pH 7, generating S3 in a dark red solid form.\(^{41}\) Compound S3 was O-alkylated by reacting 2,3,4,6-tetra-O-acetyl-alpha-D-galactopyranosyl bromide with Cs\(_2\)CO\(_3\) in ACN to give S4 in good yield.\(^{28}\) Subsequently, S4 was deprotected with NaOMe in MeOH for 1 h, yielding S5 after purifying using column chromatography (silica gel).\(^{28}\) At last, compound S5 was reacted with Ir(III) dimer Ir\(_2\)(ppy)\(_2\)Cl\(_2\) (where ppy = 2-phenylpyridine) in MeOH/DCM (1:1, v/v) overnight, followed by the addition of excess NH\(_4\)PF\(_6\), yielding complex 1 in good yield.\(^{38}\) For comparison, a complex, 2, was synthesized, which is the putative product formed after hydrolyzing the sugar moiety of 1 by β-gal. All complexes were fully characterized by \(^1\)H NMR, \(^{13}\)C NMR and HRMS (Figure 5.2). In addition, the stability of 1 was assessed by \(^1\)H NMR and UV–Vis Absorption spectroscopy (Figure 5.3).\(^{42}\) The results reveal that 1 was stable in ACN/20 mM Tris–HCl buffer with 20 mM NaCl (pH = 7.4) (8/2, v/v) or in DMSO-\(d_6\)/D\(_2\)O (9/1, v/v) over at least 3 days at 298 K. The photostability of 1 was also studied using time-based luminescence spectra.
(Figure 5.3C). No observable change in the emission of 1 over 1800 s, while a gradual decrease in the luminescence of ThT was found under the same conditions. 1 exhibited maximum excitation at 282 nm and emission at 586 nm (Figure 5.4). Other photophysical profiles of complexes 1 and 2 are summarized in Table 5.1.

**Figure 5.1.** Synthesis of complexes 1 and 2. Reagents and conditions: a) tetrabutylammonium hydrogensulfate, HClO, DCM/H$_2$O, pH 8.2–8.3; b) con. H$_2$SO$_4$, 100 °C; c) Cs$_2$CO$_3$, ACN, r.t.; d) NaOMe, MeOH/H$_2$O, rt; e) and f) Ir$_2$(ppy)$_2$Cl$_2$, NH$_4$PF$_6$, DCM/MeOH, rt.
Figure 5.2. $^1$H, $^{13}$C NMR and HRMS spectra of complexes 1 and 2.
Figure 5.3. Stability experiment of complex 1. (A) $^1$H NMR spectra of 5 mM complex 1 in the mixture of DMSO-$d_6$/D$_2$O (9/1) at 298 K over 72 h. (B) UV/Vis absorption spectra of 20 µM complex 1 in ACN/20 mM Tris–HCl buffer (pH 7.4) (8/2) containing 20 mM NaCl at 298 K over 72 h. (C) Time-based luminescence
spectra of 5 µM complex 1 and 5 µM ThT over a course of 1800 s, $\lambda_{\text{exc}}$ was set at 355 nm.

**Figure 5.4.** Maximum excitation and emission spectra of the probe in PBS (1×, pH 7).

**Table 5.1.** Photophysical profiles of complexes 1 and 2.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Quantum yield</th>
<th>$\lambda_{\text{emi}}$ (nm)</th>
<th>Lifetime (µs)</th>
<th>UV-Vis absorption $\lambda_{\text{abs}}$ / nm ($\varepsilon$ / dm$^3$ mol$^{-1}$ cm$^{-1}$) $^1$ $^a$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.10$^a$</td>
<td>586</td>
<td>3.522$^a$</td>
<td>$242 \times 10^5$, $272 \times 10^5$, $380 \times 10^4$</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>$253 \times 10^5$, $296 \times 10^4$, $377 \times 10^4$</td>
<td></td>
</tr>
<tr>
<td>2 + β-gal</td>
<td>0.05$^b$</td>
<td>552</td>
<td>3.653$^b$</td>
<td>$280 \times 10^5$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$: data was determined in ACN; $^b$: data was acquired in PBS (1×, pH 7); lifetime was measured using a standard Ru(bpy)$_3$($\text{PF}_6$)$_2$. 
The ability of 1 in response to β-gal in PBS buffer was first investigated. Notably, the luminescence of 1 enhanced significantly upon adding β-gal (Figure 5.5). There is negligible luminescence for β-gal itself, indicating the enhanced luminescence comes from the interaction between β-gal and 1. Optimization experiments were performed to optimize the conditions including PBS concentration, incubation time, complex concentration and pH value (Figure 5.6). The optimal conditions were found to be 2× PBS, 1.5 h incubation time, 2.5 µM of complex 1 and pH 7. Furthermore, 1 was incubated with different β-gal concentrations (Figure 5.7a). The luminescence of 1 increased with the concentration of β-gal at the range of 2 to 30 U/mL ($R^2 = 0.9924$), with a detection limit of 0.51 U/mL (LOD = 3σ/s), where s is the slope of the calibration curve and σ is the standard deviation of six blank signals. (Figure 5.7b). The selectivity of 1 for β-gal over other potential interfering species was also investigated. After adding 100 µM of cysteine (Cys), adenosine triphosphate (ATP), magnesium ion ($Mg^{2+}$), homocysteine (Hcy), S-adenosyl methionine (SAM), glutathione (GSH), as well as 20 µg/mL of bovine serum albumin (BSA), only negligible luminescence enhancement was observed (Figure 5.7c). Moreover, competition experiment of 1 in the co-existence of other interfering species was also investigated. The luminescence enhancement of 1 to β-gal was similar in the presence and absence of the interfering species. Based on the results, 1 is highly selective for β-gal over other interfering ions existing in cells.
Figure 5.5. The luminescence of complex $\mathbf{1}$ (5 µM), β-gal (20 U/mL), or a mixture of complex $\mathbf{1}$ (5 µM) and β-gal (20 U/mL), which were incubated at 37 °C for 1 h in PBS buffer (1×, pH 7). $\lambda_{\text{exc}} = 282$ nm.

Figure 5.6. The relative luminescence of $\mathbf{1}$ and β-gal (20 U/mL) incubated at
different (a) incubation time, (b) PBS concentration, (c) pH and (d) the concentration of complex 1. Experimental condition was 1× PBS, incubating for 1 h, pH 7, and 5 µM of 1 except where the condition was the dependent variable.

**Figure 5.7.** Luminescence responses of 1 to β-gal and other analytes. (a) Luminescence spectra of 1 in the presence of different concentrations of β-gal (2–30 U/mL). (b) The linear relationship between the concentration of β-gal and the luminescence of the complex 1/β-gal system at λ = 550 nm (c) Luminescence responses of complex 1 (2.5 µM) at λ = 550 nm to various analytes (20 µg/mL for BSA, 100 µM for other interferences). The blue bars represent luminescence of 1 in the presence of other analytes. The red bars represent luminescence of 1 containing other analytes with the further addition of β-gal (20 U/mL). λ<sub>exc</sub> was 282 nm.
To distinguish the luminescent signal of 1 to β-gal from the highly-autofluorescent background, 1 was studied in the presence of organic dyes such as rhodamine B and Cm460. When the delay time after excitation was 0 ns, a distinctive emission peak of Cm460 was found at 460 nm, which disturbed the emission of 1 to β-gal (Figure 5.8a). Encouragingly, we successfully eliminated the emission of Cm460 by setting the delay time to 333 ns. The phosphorescence of 1 treated with β-gal become more evident (Figure 5.8b). The results demonstrate that TRES could be employed to distinguish the signal of 1 for β-gal in the highly-autofluorescent biological media. Meanwhile, rhodamine B emits at 570 nm, which significantly disturbed the signal of 1 when the delay time gate after excitation pulse was 0 ns (Figure 5.8c). However, the response of 1 was dominant when the time gate was set at 333 ns delay time (Figure 5.8d). The results further reveal the potential applicability of 1 for the visualization of β-gal in the complicated samples using TRES technique.
Figure 5.8. Time-resolved luminescence spectra of 1. With the addition of Cm460 (0.1 µM) and 1 (5 µM) in the absence or presence of β-gal (30 U/mL). Delay time was set at: (a) 0 ns or (b) 333 ns; With the addition of rhodamine B (0.1 µM) and 1 (5 µM) in the absence or presence of β-gal (30 U/mL). Delay time was set at: (a) 0 ns or (b) 333 ns. The excitation wavelength was 355 nm.

The mechanism of 1 was also confirmed using high-resolution mass spectrometry (HRMS). 1 displays a peak at 859.2112 before treating with β-gal. However, a peak was found at 697.1611 after treating with β-gal, which was the molecular weight of 2 (Figure 5.9). Interestingly, the prepared complex 2 is weakly emissive without β-gal, but the luminescence intensity increased after treating with β-gal (Figure 5.10). It was proposed that the non-emissive properties of 2 were attributed to the aqueous solvent interaction, which quenches its
luminescence intensity. However, β-gal eliminates the galactose moiety of 1 and generates the dealkylated 2, which subsequently binds to β-gal. Upon binding, 2 is protected in a hydrophobic environment and become emissive through the $^3\text{MLCT}$ excited state. Other than β-gal, it is also possible that 2 may interact with other proteins (if present) e.g. BSA and trigger the luminescence enhancement (Figure. 5.10). However, 1 would only convert to 2 in β-gal, therefore, complex 1 is still highly specific for the activity of β-gal (Figure 5.7).
Figure 5.9. MALDI-TOF-HRMS of (a) complex 1 and (b) the product after complex 1 after the incubation with β-gal in PBS buffer (1×, pH 7).

![Figure 5.9](image)

Figure 5.10. Luminescence spectra of 2.5 µM complex 2 with or without β-gal (20 U/mL), and with BSA (20 µg/mL). The samples were incubated at 37 °C for 1.5 h in PBS buffer (2×, pH 7). λ_{exc} = 282 nm.

Next, the ability of 1 to discriminate ovarian cancer cells from normal cells was investigated. MTT assay was used to determine the cytotoxicity of 1 (Figure 5.11). Remarkably, 1 exhibited minimal cytotoxicity towards the normal cell line (HUVEC) and the ovarian cancer cell line (SKOV3) over 12 or 24 h, with an IC_{50} value of over 100 µM, while only slightly toxic after 48 h, with an IC_{50} values of 63.1 and 79.4 µM for HUVEC and SKOV3 cells, respectively. The results demonstrated that 1 is biocompatible under these experimental conditions for intracellular imaging.
Figure 5.11. Cell viabilities of HUVEC and SKOV3 cells in vitro after the treatment with complex 1 for indicated time: (a) 12 h; (b) 24 h; (c) 48 h.

The imaging ability of 1 for β-gal in ovarian cancer cells was also investigated. In this work, SKOV3 cells were selected as ovarian carcinoma cell line due to its high expression of β-gal. After incubating with 1 (0–60 µM) for 12 h in a CO2 incubator, the luminescence intensity of SKOV3 increased upon the increasing concentrations of 1, as shown in the confocal images (Figure 5.12). A time-course experiment was subsequently performed and successfully demonstrated the increasing luminescence of 1 over 24 h. The result is in agreement to the mechanism of luminescence enhancement (Figure 5.13). We also measured the luminescence of 1 in HUVEC cells with a quite low expression
level of β-gal. Encouragingly, only negligible luminescence was observed after incubating with 60 µM of 1 for 12 h (Figure 5.14). The result suggests that the enhanced luminescence of 1 in SKOV3 cells was due to the high expression of β-gal in cells. Importantly, there was a decreased luminescence of 1 in SKOV3 cells when preincubating the cells with a D-galactose, a β-gal inhibitor (Figure 5.15). These results indicate that the activity of β-gal in the living systems could be monitored by 1, which could be potentially employed to discriminate ovarian cancer cells and normal cells.

**Figure 5.12.** Luminescence images of SKOV3 cells treated with indicated concentrations of complex 1 (3–60 µM) for 12 h at 37 °C. Luminescence was collected between 540 and 640 nm. The excitation was set at 405 nm. The scale bar is 50 µm.
Figure 5.13. Luminescence images of SKOV3 cells after the treatment with 30 µM complex 1 for the indicated time at 37 °C. Luminescence was collected between 540 and 640 nm. The excitation was set at 405 nm. The scale bar is 50 µm.

Figure 5.14. Luminescence images of HUVEC cells after the treatment with indicated concentrations of complex 1 (3–60 µM) at 37 °C for 12 h. Luminescence was collected between 540 and 640 nm. The excitation was set at 405 nm. The scale bar is 50 µm.
Figure 5.15. Luminescence images of SKOV3 cells after the treatment with 30 µM complex 1 with or without the addition of a β-gal inhibitor D-galactose (10 mg/mL). Luminescence was collected between 540 and 640 nm. The excitation was set at 405 nm. The scale bar is 50 µm.

We further validate the ability of 1 to distinguish ovarian cancer cells from normal cells. In this work, OVCAR3, an ovarian cancer cell line, and human embryonic kidney HEK-293T, a normal cell line, were employed. Encouragingly, a high level of β-gal activity was only detected in OVCAR3, whereas substantially lower expression of β-gal was found in HEK-293T (Figure 5.16a). The results were consistent with the previous reports. We have also demonstrated that the two employed ovarian cancer cell lines (OVCAR3 and SKOV3) could be “lighted up” upon incubating with 30 µM of 1 at 37 °C for 12 h, while the normal cell lines (HEK-293T and HUVEC) only gave negligible luminescence under the same condition (Figure 5.16b). As a result, 1 demonstrates
high potential in the discrimination of ovarian cancer cell expressing a high level of β-gal and the normal cell lines.

![Figure 5.16](image)

**Figure 5.16.** Applicability of 1 to discriminate ovarian cancer cell to normal cell. (a) the β-gal activity of OVCAR3, SKOV3, HEK-293T, and HUVEC cell lines was measured by a β-gal activity kit. (b) Luminescence images of OVCAR3, SKOV3, HEK-293T, HUVEC cell lines after incubating with 30 µM of 1. Luminescence was collected between 540 and 640 nm. The excitation was set at 405 nm. The scale bar is 50 µm.

### 5.4 Conclusion

β-gal is known as a significant biomarker of ovarian cancers. Therefore, the development of sensing probes for the visualization of β-gal is of high significance for diagnosing ovarian cancer. In this work, we have conceived the
first long-lived Ir(III) complex for the discrimination of ovarian cancer cells from normal cells. The probe 1 contains a bond that could be cleaved by β-gal and gives an enhanced luminescence signal. Furthermore, 1 exhibits long lifetime and excellent stability, as well as capable of detecting β-gal activity in the highly-autofluorescent background using TRES. 1 is also highly selective toward the activity of β-gal with a linear range of 2 to 30 U/mL, and achieved a detection limit of 0.51 U/mL. Importantly, 1 could selectively “light up” ovarian cancer cells, while giving negligible luminescence in normal cells. We anticipate that 1 could potentially be employed for the diagnosis of β-gal-related diseases including ovarian cancer.
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Chapter 6

Summary and outlooks

Over the past years, organic dyes have attracted considerable attention for the development of sensing platforms for environmental analytes and disease-related biomolecules. Although organic dyes have been extensively studied, their applications are greatly limited by their low photostability, short Stokes shift, and short emission lifetime. Therefore, the development of alternative approaches overcoming these limitations is of significant importance. In this thesis, I aim to harness luminescent Ir(III) complexes to monitor environmental analytes e.g. Al\(^{3+}\) ions, as well as to visualize biomolecules e.g. the cancer biomarker β-gal and the transcription factor NF-κB in the living system. Luminescent Ir(III) complexes possess a number of attractive advantages that render them as alternative sensing probes, including long emission lifetime, high photostability, large Stokes shift, high quantum yield, ease of structural modifications, and tunable emission. Therefore, my research interest mainly lies on the incorporation of recognition units into Ir(III) complex scaffolds, which confers the ability to specially recognize target analytes, along with luminescence signal changes. In this context, I have successfully demonstrated the utility of recognition unit-conjugated Ir(III) complexes as sensitive probes for Al\(^{3+}\) ions, H\(_2\)S, hypoxia, NF-κB, and β-gal. The capability of these probes for environmental monitoring and cellular visualization has also been explored. In particular, the conjugation of endogenous molecule
galactose or natural compound oridinin to an Ir(III) scaffold to highlight the utility of these strategies to obtain probes with improved biocompatibility.

Meanwhile, although we have demonstrated the applicability of Ir(III) complexes-based imaging probes, the development of simpler synthetic strategies and procedures, as well as probes with higher biocompatibility are still desirable. Importantly, although the development of Ir(III) complex-based probes have been widely studied in recent years, the development of industrial products, such as portable in situ monitoring devices (for environmental uses) and diagnostic kits for cancer patients (for clinical uses), are still on the way. Furthermore, microfluidic chips, materials with incorporated micro-channels, has emerged as a promising technique for sensing applications. This technique sheds light on the transformation of the conventional luminescent sensing platforms into the next generation portable devices, which allows potential applications in in situ monitoring of environmental pollutants or real-time monitoring of biological analytes. Therefore, more efforts should be devoted to the integration of Ir(III) probes to microfluidic technique. Additionally, theranostics approach, which combines both the diagnostic imaging and therapy into a single utility, has also sparked considerable interests in the scientific community. Although luminescent Ir(III) complexes have achieved huge success in diagnostic and therapeutic areas, their potential applications on theranostics are not significantly explored. Therefore, there is an urgent need to explore the theranostic applicability of dual-functional Ir(III) complexes, which may aid the visualization of important
biomarkers and/or drug activities, as well as serve as a therapeutic agent at the same time.
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