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Novel Protein Staining Agents from Cationic and Neutral Luminescent Iridium(III) Complexes

Yuyang Zhou,*[a] Junli Jia,*[b] Xiaomei Wang,[a] Weiqiang Guo,[a] Zhengying Wu,[a] Nan Xu[a]

Abstract: Seven luminescent iridium(III) complexes have been deliberately prepared to thoroughly investigate the relationships between chemical structures and properties of protein staining in this work. It's the first time to reveal the effect of main ligand, π conjugation effect of ancillary ligand and charge effect of organometallic complexes on protein staining. Most importantly, this study gives the first experimental evidence of the potential applications of charged-neutral organometallic complexes in protein staining, which could open an avenue of exploiting novel protein staining agents in the future.

Luminescent properties are always one of the major research areas for organometallic complexes, including iridium(III),[1] ruthenium,[2, 3] rhenium,[4, 5] copper,[6] platinum,[7, 8] etc. Due to comparatively high quantum efficiency, excellent anti-photobleaching ability, large Stokes' shift and fine emission tunability, iridium(III) complexes are much more attractive and exhibit competent abilities in widespread areas, such as phosphorescent organic light-emitting diodes (PhOLEDs),[9] electrochemiluminescence (ECL),[10, 11] chemiluminescence[12] and biological sensors.[13-17]

Beside the above-mentioned areas, one of the important applications of luminescent organometallic complexes is protein staining which is usually one of the prominent procedures in the proteomic analysis.[18, 19] Unfortunately, except the commercial product named SYPRO Ruby,[20] the researches on protein staining of luminescent organometallic complexes are extremely rare until now and the development on new class of protein staining agents is also very slow in the past few years. A notable example is Wong et al reported a non-emissive iridium(III) complex could selectively stained histidine/histidine-rich proteins based on luminescent switch-on mechanism through replacement reaction of coordination ligands.[21] Based on the same reaction model, Li et al synthesized a non-emissive complex $[Ir(ppy)_2(DMSO)_2]_+PF_6$ to specifically light up the nuclei of living cells.[22] However, this type of staining agents would covalently bind with the histidine residues in protein, which makes it incompatible with the downstream mass spectrometric analytical technics for further protein identification in proteomics. Another typical work is a series of luminescent iridium(III) with bathophenanthroline disulfonate (abbreviated as BPS) group has been reported as protein staining agents, which is inspired by the chemical structure of the commercial staining agent of ruthenium(II) tris(bathophenanthroline disulfonate) (abbreviated as RuBPS).[23] However, it's tough to incorporate the functional group of BPS into the coordination ligands and the purification of the corresponding iridium(III) agents is also very difficult due to regional-isomers of BPS on ancillary ligand,[24] which hindered its potential commercialization in the future. Moreover, it should be noted that Che group developed a novel platinum complex for practical application in protein staining as well.[25] In addition, a ruthenium complex has also been reported as a switch-on probe to BSA and histidine-tagged proteins through non-covalently reaction model by Wong et al.[26] In recent years, based on G-quadruplex motif,[27] a number of iridium(III) complexes have also been developed as the luminescent probes to enzymes.[15,16,28,29] Although these reports have made contributions to the development of novel staining agents, there is still rare of detailed studies about the relationships between chemical structures and properties of protein staining.

Herein, a series of iridium(III) complexes containing 4,7-diphenyl-1,10-phenanthroline (DIP) ancillary ligand (**1**-**4** in Figure 1) have been designed and synthesized by changing the main ligands in this work. In the further studies about the relationship between chemical structure and the properties of protein staining, bis(2-phenylpyridine-C^2,N)(2,2-bipyridyl)iridium(III)chloride [named **Ir(ppy)$_2$bpy**] and two electronically neutral complexes, i.e. tris(2-phenylpyridine-C^2,N)iridium(III) [named **Ir(ppy)$_3$**] and tris(2-phenylquinoline-C^2,N)iridium(III) [named **Ir(pq)$_3$**] have also been selected as control compounds to carry out the experiments of protein staining. The chemical structures of iridium(III) complexes used in this work have been shown in Figure 1. In this communication, the π conjugation effect of coordination ligands and charging effect of iridium(III) complexes on protein staining have been investigated thoroughly for the first time. In addition, the living cell imaging and cytotoxicity assays of complexes **1**-**4** with DIP ligand have also been studied in this work.

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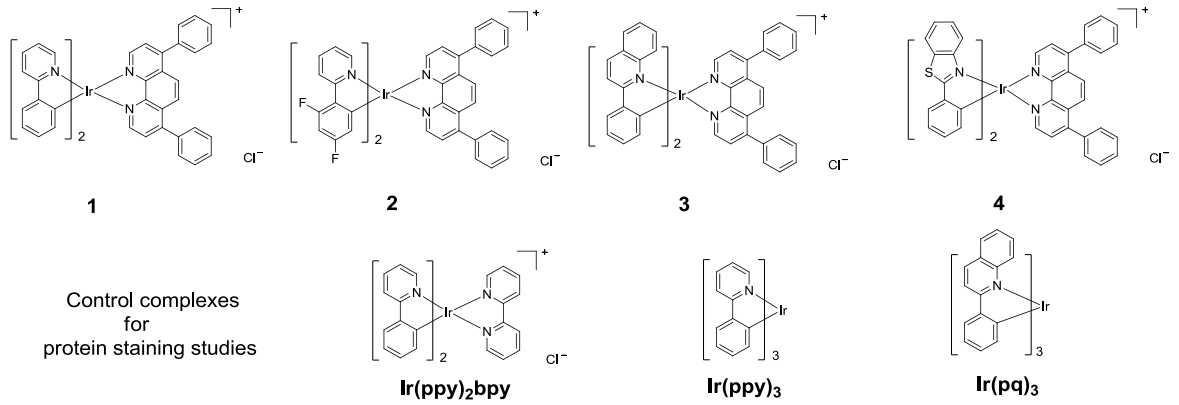

Figure 1. The chemical structures of iridium(III) complexes used in this work.

The general synthetic routes (Scheme S1 in Supporting Information) and chemical structure characterization of complexes **1**-**4** have been shown and described in Supporting Information and no discussion is needed here. Taking into account the absorption and emission data of three control compounds, i.e. **Ir(ppy)$_2$bpy**, **Ir(ppy)$_3$** and **Ir(pq)$_3$**, have been reported in the previous literatures, [11, 30, 31] the photophysical results of complexes **1**-**4** with DIP ligands have just been shown (Figure 2 and Table S1) and discussed in this work. According to Figure 2A, the absorption band of complexes **1**-**4** with DIP ancillary ligand mainly located in the range of blew 500 nm, which is identical to other reported iridium(III) complexes.[10, 32-39] Generally, the absorption band between 350-500 nm is assigned to the state of metal to ligand charge transfer (MLCT), for which the molar coefficient (ε) is below 1×10^4 $M^{-1}cm^{-1}$, while the band below 350 nm (ε $>10^4$ $M^{-1}cm^{-1}$) is ascribed to the state of intra ligand charge transfer (ILCT). It should be noted that complex **2** has slightly weak absorption intensity compared with complexes **1**, **3** and **4**, which is reasonably explained that the electron-withdrawn substituent of –F in complex **2** would decrease the electron density. Meanwhile, compared with complex **1**, complex **3** with larger conjugation degree in the main ligand have a little more intense absorbance.

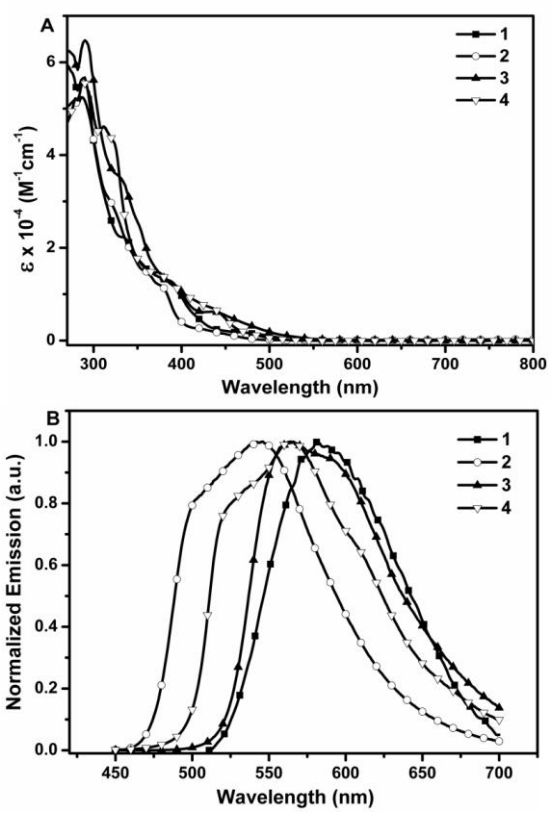

Figure 2. The absorption (A, 40 μM) and normalized emission spectra (B, 8 μM) of **1**-**4** in dichloromethane solution. ε is the molar extinction coefficient.

Figure 2B shows the normalized emission spectra of complexes **1**-**4** in dichloromethane. Unlike the slight distinction in absorption spectra, as expected, there is much difference in emission spectra due to the variation of coordination main ligands. As shown in Figure 2B, it's interesting to find that beside the main emission peak, complexes **2**, **3** &**4** also have other shoulder peaks, which have also been listed in Table S1 in Supporting Information. This phenomenon of shoulder peak have also been observed in some other iridium(III) complexes. [10, 33, 40] Moreover, using the *fac*-Ir(ppy)$_3$ as the reference (Φ_{PL}=40%),[31] the quantum efficiencies of these complexes in degassed dichloromethane are also calculated and listed in Table S1. The Φ_{PL} is variable due to the changing of main ligands and follows the sequence: **4** > **2** > **3** > **1**. Through optimizing chemical structures of the main ligands, three novel iridium(III) complexes, named **2**, **3** and **4** in this work, all exhibit higher quantum efficiency than complex **1** and the Φ_{PL} of complex **4** is up to 46.3%. The high quantum efficiency demonstrated that these iridium(III) complexes in this work have excellent luminescent properties and have potential applications of protein staining and living cell imaging.

To evaluation the utilities of these luminescent iridium(III) complexes in protein staining, one-dimensional gel electrophoresis of seven mixed, unstained protein molecular weight (M_w) marker has been chosen as our evaluation system in this study. The commercial protein marker used in this study includes seven native proteins with variation M_w, that is β-galactosidase (β-gal, 116.0 KDa), Bovine serum albumin (BSA, 66.2 KDa), Ovalbumin (Ova, 45.0 KDa), Lactate dehydrogenase (Lac de, 35.0 KDa), REase Bsp981 (REase, 25.0 KDa), β-lactoglobulin (β-lac, 18.4 KDa) and Lysozyme (Lys, 14.4 KDa). The staining protocol has well been described in the Supporting Information. It should be noticed that these iridium(III) complexes reported in this work don't need de-staining step because there is no reduction of background noise signals when

a 24 hours de-staining step was employed, which is more timesaving than commercial products of SYPRO Ruby, Coomassie Brilliant Blue dye and colloidal-silver staining agents.[26] Figure 3 shows the emissive gel image containing commercial protein marker after staining with 1 μM luminescent iridium(III) complexes for 6 hours. The protein amounts are diluted sequently in half from left lane to right lane in each gel. The limits of detection (LOD) of these staining agents for different proteins have also been listed in Table S2 in Supporting Information. Obviously, along with the decrease of protein amounts, the fluorescent intensities of these bands reduced from left to right accordingly and the LOD of these iridium(III) staining agents is lower to the level of several nanograms (ng) (see Table S2 in Supporting Information). Reasonably, these staining agents displayed variable performance due to the changing of chemical structures. As the alternatives of the commercially protein staining agents, the lower of protein to protein variations would mean wider applicability in staining different proteins, which is much desirable and more competitive in the real markets. Herein, the protein-to-protein variations of these staining agents have also been charted as Figure 4 according to the data collected from the imaging bands of Figure 3 in this work. In the following, the protein staining results would be discussed in details.

First of all, for the complexes **1**-**4**, it's the primary focus to investigate the effect of main ligand on protein staining. According to Figure 3, either increasing π conjugation or incorporating –F group would not induce the fail of staining. The differences among them are mainly on their LODs to different proteins. Compared complex **1** with complex **3** and **4**, no improved performance of protein staining was found through increasing π conjugation degree of main ligand. Meanwhile, incorporating two –F groups into the main ligand from complex **1** to complex **2** would increase the LOD of the staining agent to the proteins with lower molecular weight (β-lac and Lys, see Figure 3 and Table S2).

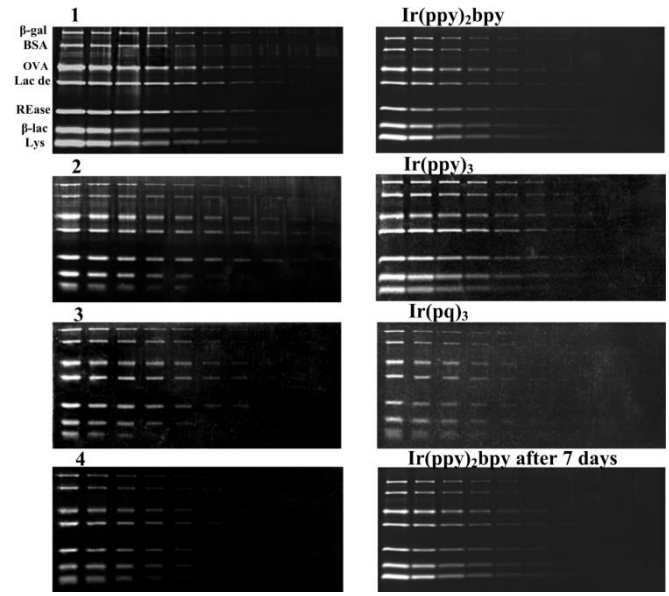

Figure 3. Images of protein bands stained with **1**-**4** and three control complexes after SDS-PAGE. The commercial protein marker used in this work includes β-galactosidase (β-gal, 116.0 KDa), Bovine serum albumin (BSA, 66.2 KDa), Ovalbumin (Ova, 45.0 KDa), Lactate dehydrogenase (Lac de, 35.0 KDa), REase Bsp981 (REase, 25.0 KDa), β-lactoglobulin (β-lac, 18.4 KDa), Lysozyme (Lys, 14.4 KDa).

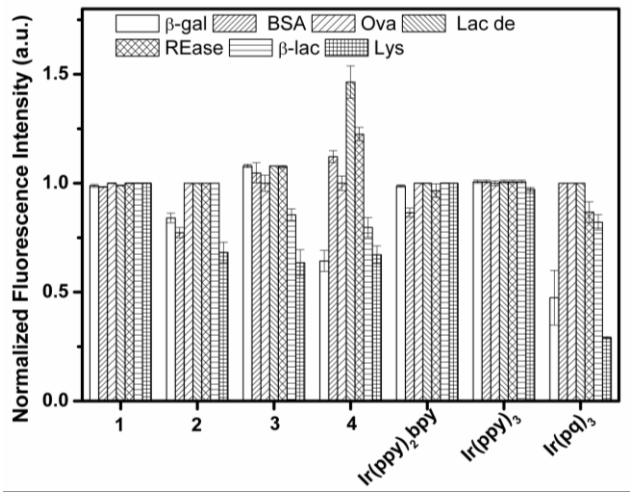

Figure 4. Protein-to-protein variations of the luminescent iridium(III) staining agents. Normalized to the fluorescent intensity of Ovalbumin.

To the best of our knowledge, although some luminescent organometallic complexes have been reported as protein staining agents, the relationships between chemical structures and protein staining have not been thoroughly studied so far. Herein, beside the above studies about the effects of main ligand, the further research about the π conjugation effect of ancillary ligands on protein staining has also been carried out. As shown in Figure 1, containing the same ancillary ligand of DIP is the common feature of complexes **1**-**4**. In order to test the function of DIP in protein staining, the first control compound named **Ir(ppy)$_2$bpy** (Figure 1) has been prepared by replacing DIP ligand of complex **1** with 2,2'-bipyridine (bpy). According to Figure 3, **Ir(ppy)$_2$bpy** still displayed comparable capabilities of protein staining though decreasing the π conjugation degree of ancillary ligand, which indicates the large π conjugation degree of DIP is not necessary to the design of protein staining agents.

Another key factor in protein staining is usually identified as the electrostatic forces between staining agents and protein, thus ionic organometallic complexes are deliberately designed as protein staining agents in the previous studies.[25, 41] In addition, a class of cationic iridium(III) complexes with diimine ligand have also been patented by Berkelman.[42] However, to the best of our knowledge, there is no report about electronically neutral complexes as protein staining agents up to now. In order to reveal the charge effect of luminescent organometallic complexes on protein staining, **Ir(ppy)$_3$** and **Ir(pq)$_3$** (see Figure 1) as the electronically neutral complexes were further selected to carry out another control experiments of protein staining in this work. As shown in Figure 3, it's surprising to find that even neutral iridium(III) complexes have staining ability as well. These findings give the direct experimental evidences of the potential applications of charged-neutral complexes in protein staining.

In addition, the stability of these staining agents is also one of the important issues in the real applications. It should be emphasized that there is no obviously reduction of the intensities of the imaging bands after keeping the stained gels in deionized water for one week at room temperature. A typical image of gel

stained by **Ir(ppy)$_2$bpy** after 7 days has also been shown in Figure 3, which indicates these iridium(III) staining agents have good stabilities.

Moreover, commercial staining agent of SYPRO Ruby has also been performed as positive standard to evaluate the properties of these iridium(III) agents. The image of protein bands after SDS-PAGE and the LOD are shown in Figure S1 and listed in Table S2, respectively. Accordingly, we can safely assumed that these iridium(III) staining agents are equivalent or even better than the commercial agent of SYPRO Ruby in terms of LOD. Beyond LOD, the staining protocol of these iridium(III) agents is also simpler than that of SYPRO Ruby because there is no troublesome of de-staining step for these iridium(III) staining agents.

Last but not the least, after carefully investigating the properties of these iridium(III) staining agents, including LOD and protein to protein variations, it's interesting to find that staining agents containing 2-phenypyridine (ppy), i.e. cationic complex **1**, **Ir(ppy)$_2$bpy** and charged-neutral complex **Ir(ppy)$_3$** outperformed to other iridium(III) staining agents. Increasing π conjugation of coordination ligand, either main ligand (from complex **1** to complex **3**) or ancillary ligand (**Ir(ppy)$_2$bpy** to complex **1**) would not obviously improve the performance of staining agents. Even the charged-neutral complex, for example **Ir(ppy)$_3$**, also have excellent performance of protein staining. Though the cationic staining agents is better than its corresponding charged-neutral form in the point of LOD (for example, comparing **1** with **Ir(ppy)$_3$** or comparing **3** with **Ir(pq)$_3$**), we can safely make a conclusion that the electrostatic forces may play some effect in the staining process, but not the decisive factor. As for the reasons of staining mechanism for these agents, as we know, these iridium(III) complexes are hydrophobic[39] and the proteins in the gel after SDS-PAGE are also hydrophobic,[23] which may be the direction of explaining their binding affinities.

Beyond protein staining, we have also extended the potential applications of cationic luminescent complexes **1**-**4** into living cell imaging. The luminescent images of HeLa cells co-incubated with 20 μM iridium(III) complexes for 1 hours at 37 °C has been shown in Figure S2 in Supporting Information, which shows that the intracellular distribution of complexes **1**-**4** with DIP ligand is mainly in the cytoplasm, rather than nucleus and membrane. Furthermore, the cytotoxicity assays of complexes **1**-**4** towards three cancer cell lines (HeLa, A549, MCF-7) are performed and the IC_{50} data has also been listed in Table S3 in Supporting Information. The lower IC_{50}s of complexes **1**-**4** toward cancer cell lines indicate complexes **1**-**4** have anticancer activities as well. All these results demonstrated these cationic iridium(III) complexes with DIP ligand have potential applications in bio-imaging or emerging cancer therapeutics beyond protein staining.

In conclusion, seven luminescent iridium(III) complexes have been synthesized and investigated their performances in protein staining and living cell imaging. Most importantly, beside the effect of main ligand, the π conjugation effect of ancillary ligand and charge effect of iridium(III) complexes on protein staining have also been studied by carrying out experiments using three prominent control compounds named **Ir(ppy)$_2$bpy**, **Ir(ppy)$_3$** and **Ir(pq)$_3$** as protein staining agents. These iridium(III) staining agents have equivalent or even better performance than the commercial agent of SYPRO Ruby both in terms of LOD and time cost. It's the first time to thoroughly reveal the relationships between chemical structure of luminescent iridium(III) complexes and the performance of protein staining. Furthermore, this work gives the first experimental evidence of the potential applications of charged-neutral complexes in protein staining, which opens an avenue to exploit novel protein staining agents in the future.

Supporting Information

The synthetic routes and characterization of iridium(III) complexes; experimental details for protein staining, living cell imaging and cytotoxicity assay; the summarized photophysical properties of **1**-**4**; the protein staining results from commercial agent of SYPRO Ruby; The limits of detection (LOD) of iridium complexes and SYPRO Ruby for different proteins; Fluorescence images of HeLa cells incubated with complexes **1**-**4** for 1 hour at 37 °C; Cytotoxicity of complexes **1**-**4** and NMR spectra.

Acknowledgements

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Keywords: iridium complex • protein staining • neutral • luminescent • organometallic

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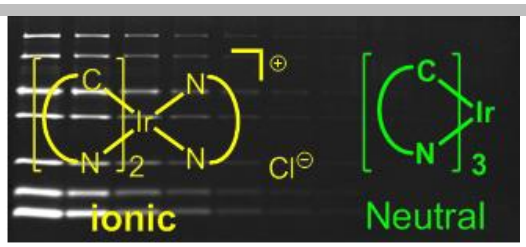
COMMUNICATION

It's the first time to justify the application of charged-neutral iridium(III) complexes in protein staining beyond the cationic iridium(III) complexes.



Yuyang Zhou, Junli Jia,* Xiaomei Wang, et al.*

Page No. – Page No.

Novel Protein Staining Agents from Cationic and Neutral Luminescent Iridium(III) Complexes