Synthesis and Structural Optimization of Iridium(III) Solvent Complex for Electrochemiluminescence Labeling of Histidine-Rich Protein and Immunoassay Applications for CRP Detection

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ABSTRACT: The reaction between an iridium(III) solvent complex and the histidine site of biomolecules as one kind of novel bioconjugation approaches has received much attention during the past few years. To extend this novel bioconjugation approach into electrochemiluminescence (ECL) immunoassay and optimize the performances, three iridium(III) solvent complexes with different C^N bidentate main ligands have been designed and synthesized in this work. Bovine serum albumin (BSA) as the standard histidine-rich protein is initially employed to evaluate the labeling performances by comparing the ECL intensity of the same amount of BSA labeled by different iridium(III) solvent complexes. Importantly, a magnetic beads-based sandwich immunoassay platform using Ir-dmpq (iridium(III) acetonitrile complex with 2-(3,5-dimethylphenyl)quinoline as the main ligand) as a structurally optimized labeling agent has been successfully constructed to detect C-reactive protein (CRP, an important biomarker of systemic inflammation in clinic), and the limit of detection based on this novel labeling agent could reach below 1 ng/mL, which may further pave the way for applications of the iridium(III) solvent complex in histidine-rich protein ECL labeling beyond fluorescence labeling.

INTRODUCTION

Developing novel luminescence labeling approaches and corresponding excellent luminescent labels is always one of the significant issues in biochemistry and related fields. In the past few decades, owing to their excellent luminescence properties, iridium(III) complexes have received much attention and have been developed into reliable luminescent probes for various biomolecules. Notably, one kind of nonemissive iridium(III) solvent complex, Irppy_2(MeCN)_2^+, (ppy is 2-phenylpyridine, MeCN demonstrates acetonitrile, Irppy in this work), was initially reported as a fluorescent switch-on probe for histidine and histidine-rich proteins by Ma et al. The revealed mechanism is that the solvent ligand of the original iridium(III) complex could be substituted with histidine and a new emissive histidine-bound iridium(III) complex is on-site re-formed accordingly. The possible coordination types between histidine and the iridium(III) center have also been further demonstrated in detail by Wang et al. Meanwhile, Li et al. synthesized a dimethyl sulfoxide (DMSO)-coordinated iridium(III) complex, Irppy_2(DMSO)_2^+, to specially light up nuclei of living cells based on the proposed histidine-binding mechanism. Subsequently, this novel bioconjugation method demonstrated various applications, such as imaging of subcellular organelles, multiplex assays of cell death, differentiating free amino acids from biomolecules containing the same amino acids, and luminescence labeling of cyclized peptides. However, these studies are all dependent on the changes of photo-luminescent (PL) signals specifically occurring upon binding of histidine residues to an iridium(III) solvent complex.

Electrochemiluminescence (ECL) is defined as the emission of light generated on an electrode through the electrochemical formation of excited species. Due to the advantages of high sensitivity, low noise, and signal specificity, ECL-based analytic systems have achieved great success in clinical diagnostic markets currently. However, taking into account the disadvantages of the low quantum efficiency and hardly tunable emission colors for the ruthenium-based labels widely used in the commercial equipment, novel kinds of non-ruthenium metal complexes are extensively explored for ECL applications in the academic field, including iridium(III) complexes, platinum(II) complexes, and others. Though novel ECL luminophores have achieved great advances in the past few years, there is still scarcity of innovations in the bioconjugation approaches between ECL luminophores and biomolecules.

Taking into account the advantages of ECL over PL in analytical applications, we initially investigated ECL signal changes upon the addition of histidine to the solution of Ir(ppy)_2(MeCN)_2^+ and developed Ir(ppy)_2(MeCN)_2^+ as an
efficient and specific ECL probe for histidine over other amino acids. This study directly proved that the ECL signals of histidine-bound iridium(III) complexes could be obviously detected in commercially available ECL buffers. Very recently, Zhou and Xia have shown ECL immunoassay applications of the solvent complex Ir(ppy)$_2$(MeCN)$_2$ as a novel protein ECL labeling agent, which further strongly demonstrated more attractive prospects of iridium(III) solvent complexes in ECL labeling applications.

After reviewing the related literature, this innovative labeling method is almost entirely limited to the original iridium(III) complex with 2-phenylpyridine ligand as the main ligand, such as Ir(ppy)$_2$(MeCN)$_2$, Ir(ppy)$_2$(H$_2$O)$_2$, and Ir(ppy)$_2$(DMSO)$_2$. Obviously, the following labeling performances (including emission color and quantum efficiency) largely relied on the on-site-formed emissive complex of Ir(ppy)$_2$(His)$_2$. (His represents the histidine residues of the biomolecules, such as peptides and proteins). It is well known that the emission color and quantum efficiency are seriously related to the main ligand of the iridium(III) complex. Specifically, the luminescence performances of the on-site-formed iridium(III) complex, i.e., Ir(C$^\wedge$N)$_2$(His)$_2$, could be optimized by changing the main ligand of the original complex of Ir(C$^\wedge$N)$_2$(solvent)$_2$ (solvent is MeCN, H$_2$O, or DMSO). Herein, three iridium(III) solvent complexes with different C$^\wedge$N bidentate ligands (shown in Figure 1) have been rationally designed and successfully synthesized in this work. The structure design principle, photophysical properties, and the frontier orbital calculations have been thoroughly characterized. Most importantly, the ECL performance and the following magnetic bead-based sandwich immunoassay for the C-reactive protein (CRP) detection have been investigated in this work. The structurally optimized label would further promote the development of innovative histidine-binding labeling techniques in expanding related fields.

## RESULTS AND DISCUSSION

### Design and Synthesis

Taking into account the dramatical needs of multicolor labels in the further developments of analytical techniques, ECL labels with tunable colors came to our major concerns in designing chemical structures of iridium(III) solvent complexes. According to the revealed relationships between the luminescence properties and chemical structures of iridium(III) complexes, the strategies of adjusting the conjugation degree and incorporating substituents with different electronic effects are finally adopted in this work. As shown in Figure 1, 2-phenylpyridine (ppy), 2-(2,4-difluorophenyl)pyridine (dfppy), and 2-(3,5-dimethylphenyl)quinoline (dmpq) have been selected as the C$^\wedge$N main ligand to design highly efficient ECL dyes in our former studies, which encouraged us to further synthesize the corresponding solvent complex of Ir-dmpq in this work. Notably, though new iridium(III) complexes would be re-formed through solvent ligand substitution of histidine-rich biomolecules in the following labeling applications, these selected main ligands still remained in the skeleton of the re-formed luminophores and the strategy used in this work remained reasonable.

The synthesis procedures of these complexes are detailed in the Experimental Section. Briefly, the dichloro-bridged iridium(III) dimer is first obtained by the reaction of iridium(III) chloride hydrate and the C$^\wedge$N main ligand, while the next step is to synthesize the corresponding acetonitrile coordinated iridium(III) complexes under the assistance of AgBF$_4$ in acetonitrile solutions. Finally, NMR (Figures S1–S6 in the Supporting Information) and time-of-flight mass spectra (TOF-MS) (Figures S7–S9 in the Supporting Information) characterizations have been used to identify the chemical structures of these iridium(III) solvent complexes. Notably, due to the low forces of the coordination band of acetonitrile in these labels, there are no molecular ion peaks recorded for Ir-dmpq and Ir-ppy, while the fragmental peaks at 698.2123 and 501.0930 should be reasonably ascribed to the decomposition of one acetonitrile ligand and two acetonitrile ligands for the cationic part of Ir-dmpq and Ir-ppy, respectively. Fortunately, the molecular ion peak of Ir-dfppy
located at 655.1072 has been successfully detected though the intensity is very weak.

**Photophysical Properties.** The photophysical properties have been thoroughly investigated before the ECL-based analytical applications. Figure 2A shows the absorption spectra of these iridium(III) solvent complexes in acetonitrile solution at room temperature. Most importantly, the quantum efficiency is also calculated by referring to the standard sample of fac-Ir(ppy)$_3$ ($\Phi_{PL} = 0.40$). All critical photophysical data are also listed in Table 1. According to Table 1, the photoluminescence of these iridium(III) solvent complexes is very weak ($\Phi_{PL} \approx 1\%$). The normalized PL spectra as presented in Figure 2B confirmed that the emission color could be rationally adjusted by changing the main ligand of iridium(III) solvent complexes, which encouraged us to further carry out the following studies and analytical applications.

**Theoretical Calculations.** To understand the photophysical properties, density functional theory (DFT) and time-dependent density functional theory (TD-DFT) with B3LYP are further employed to investigate the frontier orbitals of ground states and excited states of these iridium(III) solvent complexes. In this work, the LANL2DZ basis set is adopted for the iridium(III) atom, while the 6-31G basis set is used for other nonmetal atoms, such as C, N, H, O, and F atoms.

Table 2 shows the electron distributions and the corresponding energy levels of critical frontier orbitals on the optimized ground state of iridium(III) solvent complexes in this work. Overall, for the highest occupied molecular orbital (HOMO), lowest unoccupied molecular orbital (LUMO), and the adjacent frontier orbitals listed in Table 2, there are very few electron distributions on the solvent coordination ligand part of all of the three iridium(III) solvent complexes, while concentrated electron distributions are located on the part of the C\(^{\text{N}}\) main ligand and the central iridium(III) atom, which reasonably explained the significant role of the main ligand of iridium(III) complexes in tuning the photophysical properties. In comparison with Ir-ppy, the electron-withdrawing substituent of $\text{--F}$ on the phenyl ring of Ir-dfppy would stabilize both HOMO and LUMO. However, due to the much larger impact on the energy level of the HOMO rather than the LUMO, the combined effect of $\text{--F}$ makes the energy gap between the HOMO and LUMO ($\Delta E$) increase from 3.89 eV of Ir-ppy to 4.14 eV of Ir-dfppy. This theoretical calculation result is also well consistent with the observed PL experimental phenomenon of a slight hypsochromic shift of the main peaks from Ir-ppy to Ir-dfppy in Figure 2B. However, increasing the $\pi$ conjugation degree from Ir-ppy to Ir-dmpq has yet an opposite effect on HOMO and LUMO. In detail, through destabilization of the HOMO and stabilization of the LUMO, the energy gap between HOMO and LUMO ($\Delta E$) decreased dramatically from 3.89 eV of Ir-ppy to 3.07 eV of Ir-dmpq. Accordingly, the bathochromic shift of the PL spectra from Ir-ppy to Ir-dmpq has also been well observed, which further indicated that the theoretical calculation methods used in this work are quite reliable.

Moreover, the excited states of these iridium(III) solvent complexes are also investigated by TD-DFT calculations. Table 3 lists the calculated transition wavelength, oscillator strength, and molecular orbitals involving the lowest-energy transition of

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**Table 1. Summarized Photophysical Data and Lipophilicity of Iridium(III) Solvent Complexes in This Work**

| Complex | $\lambda_{abs}$/$\text{nm}$ ($\epsilon$/$10^4$ M$^{-1}$ cm$^{-1}$) | $\lambda_{max}$/$\text{nm}$ ($\epsilon$/$10^4$ M$^{-1}$ cm$^{-1}$) | $\Phi_{PL}$ (%) | Lifetime ($\mu$s) | Log $P_{ow}$ |
|---------|-----------------|-----------------|-------------|-----------------|---------------|
| Ir-ppy  | 248(5.00); 262 (4.37); 294 (2.17); 372 (0.57); 396 (0.39) | 470; 502; 533 | 0.96 | 7.74 | $-1.138$ |
| Ir-dfppy | 241 (4.92); 255 (4.34); 309 (1.67); 352 (0.62) | 462; 480; 509 | 0.94 | 8.16 | $-0.351$ |
| Ir-dmpq | 242 (3.68); 264 (3.87); 285 (3.20); 343 (0.71); 359 (1.70); 429 (0.60) | 592 | 1.32 | 7.73 | $-0.667$ |

$^a$Nitrogen-saturated acetonitrile solution. $^b$Referred to fac-Ir(ppy)$_3$ ($\Phi_{PL} = 0.40$).
these iridium(III) solvent complexes. In combination with electron distributions on the frontier orbitals as shown in Table 2, the singlet and triplet states of these iridium(III) solvent complexes should be assigned to the mixed states of intraligand (C=N main ligand) charge transfer (ILC=NNT) and metal-to-ligand (C=N main ligand) charge transfer (MLC=NNT).

According to the results of theoretical calculations, acetonitrile as the solvent ligand has less effect on the excited states of iridium(III) complexes in this work. In summary, theoretical calculation results further proved that adjusting the chemical structures of the main ligands should be an effective approach to tune the photophysical properties of these iridium(III) solvent complexes.

**ECL Labeling Applications.** Before labeling applications, the lipophilicities (log P_o/w) of these iridium solvent complexes have been measured and are listed in Table 1. It is found that all of these cationic complexes are hydrophilic. Furthermore, incorporating two fluoride atoms induced the hydrophobicity of Ir-dfppy increasing. According to the labeling mechanism of the iridium(III) solvent complex, the ECL signal is finally related to the histidine-binding iridium(III) complex. Herein, to reasonably investigate the ECL labeling properties of these iridium(III) solvent complexes, bovine serum albumin (BSA) with rich histidine residues is initially selected as the standard protein to evaluate the properties of these novel labeling agents based on the ECL intensity of BSA labeled using iridium(III) solvent complexes. As shown in Figure 3A, the same amount of BSA labeled using different iridium(III) solvent complexes displays distinct ECL intensity. According to the intensity of the ECL signal, the labeling of Ir-dmpq outperforms that of Ir-ppy and Ir-dfppy. Subsequently, the ECL spectra are also characterized and listed in Figure 3B. It should be noted that there are no valid spectral data acquired from the BSA labeled by Ir-dfppy (the concentration of BSA is 0.6 mg/mL) due to the very low ECL intensity in the commercial ECL buffers. Meanwhile, the BSA labeled by Ir-ppy and Ir-dmpq obviously exhibited green and red emissions centered at 488 and 618 nm, respectively, which indicates that the final ECL performances (including the intensity and emission wavelength) of the labeled biomolecules could be adjusted by the main ligand of the C=N coordination ligand. These multicolor labeling results

![Image of Table 2](https://dx.doi.org/10.1021/acsomega.9b04159)

**Table 2. Electron Distributions and Energy Levels of Frontier Orbitals on the Optimized Ground State of Iridium(III) Solvent Complexes in This Work**

| Complex | HOMO-1 | HOMO | LUMO | LUMO+1 |
|---------|--------|------|------|--------|
| Ir-ppy  |        |      |      |        |
|         | -8.45 eV | -7.93 eV | -4.04 eV | -3.99 eV |
| Ir-dfppy|        |      |      |        |
|         | -8.92 eV | -8.56 eV | -4.42 eV | -4.37 eV |
| Ir-dmpq |        |      |      |        |
|         | -8.17 eV | -7.48 eV | -4.41 eV | -4.23 eV |

![Image of Table 3](https://dx.doi.org/10.1021/acsomega.9b04159)

**Table 3. TD-DFT Calculation Results of the Excited States of Iridium(III) Solvent Complexes in This Work**

| Complex | State | λ<sub>max</sub> (nm) | f  | Assignments |
|---------|-------|----------------------|----|--------------|
| Ir-ppy  | T<sub>1</sub> | 450                  | 0.0000 | HOMO → LUMO (0.53896) |
|         |        |                      |     | HOMO - 1 → LUMO + 1 (−0.31669) |
|         |        |                      |     | HOMO - 2 → LUMO (0.19736) |
| Ir-dfppy| T<sub>1</sub> | 429                  | 0.0000 | HOMO → LUMO (0.69799) |
|         |        |                      |     | HOMO - 1 → LUMO + 1 (0.37336) |
|         |        |                      |     | HOMO - 2 → LUMO (−0.22787) |
| Ir-dmpq | T<sub>1</sub> | 570                  | 0.0000 | HOMO → LUMO (0.64658) |
|         |        |                      |     | HOMO - 2 → LUMO + 1 (−0.20056) |
|         |        |                      |     | HOMO - 3 → LUMO (0.12167) |
|         |        |                      |     | HOMO → LUMO (0.70147) |
are also in good accordance with the original design principles of the chemical structures of these labels.

It is well known that the ECL intensity is usually highly related to the electrode potential. Herein, to investigate the relationship between the electrode potential and ECL intensity of BSA labeled by these novel agents, various potentials from 1 to 1.8 V were applied on the glassy carbon electrode and the ECL signals have been characterized accordingly. As shown in Figures S10−S12 in the Supporting Information, 1.4 V vs Ag/AgCl is the best potential for the labels of Ir-ppy and Ir-dmpq; meanwhile, the best potential is 1.6 V vs Ag/AgCl for the agent of Ir-dfppy. These results indirectly revealed that the re-formed ECL luminophores with different main ligands exhibit different redox behaviors.

The excellent performance of Ir-dmpq in BSA labeling studies encouraged us to further implement its real application of labeling clinical biomarkers. C-reactive protein (CRP) is a pentameric plasma protein with homologs in vertebrates and many invertebrates that participate in the systemic response to inflammation.30 Currently, CRP is clinically recognized as an important biomarker for systemic inflammation. Herein, the magnetic bead-based sandwich ECL immunoassay of CRP using Ir-dmpq as the ECL labeling agent has been deliberately designed, and the schematic graph of the immunoassay is also shown in Figure 4. Before employing the sandwich immunoassay as shown in Figure 4, Ir-dmpq as the labeling agent is initially incubated with CRP antibody for 3 h and the CRP antibody labeled with Ir-dmpq is obtained after dialysis (removing the redundant labeling agent). Then, sandwich immunocomplexes are formed on the surface of the magnetic beads through three major steps as illustrated in Figure 4. Finally, after carefully transferring the immunocomplexes into the ECL measurement cell coupled with the glassy carbon working electrode, ECL signals are successfully generated under the assistance of commercial ECL buffer containing tri-n-tripropylamine (TPA) as the co-reactant.

Based on the typical sandwich immunoassay platform as shown in Figure 4, different concentrations of CRP (antigen) have been employed to evaluate the properties of the Ir-dmpq labeling agent in real-time applications. As can be seen in Figure 5A, the intensity of ECL signals increased along with the increase of CRP (antigen) concentration from 0 to 600 ng/mL. The integrated ECL intensity is linear with the logarithm of the CRP concentration (as shown in Figure 5B). At a signal-to-noise ratio of 3, the limit of detection of the sandwich immunoassay based on Ir-dmpq as the labeling agent could reach below 1 ng/mL. These preliminary results demonstrated that Ir-dmpq as one kind of novel ECL labeling agent has attractive prospects, and thus further optimization and applications of ECL immunoassay based on Ir-dmpq as a novel labeling agent are underway in our laboratory.

CONCLUSIONS

In conclusion, three iridium(III) solvent complexes with different main ligands have been rationally designed and successfully synthesized in this work. Through changing the main ligands, these three iridium complexes in the following ECL labeling applications displayed various emission colors and ECL intensities in commercially available buffers. Furthermore, Ir-dmpq as the most efficient novel red ECL label has also been employed to detect CRP (an important biomarker of inflammation in clinic) based on the typical sandwich immunoassay platforms and the limit of detection could reach below 1 ng/mL. Taking into account the color

Figure 3. (A) ECL intensity of 0.6 mg/mL BSA labeled using different iridium(III) solvent complexes in ProCell solutions. The applied potential is 1.4 V vs Ag/AgCl, the acquired time is 20 s, ProCell buffer (containing tri-n-propylamine). (B) ECL spectra of BSA labeled by Ir-ppy and Ir-dmpq.

Figure 4. Schematic graph of sandwich immunoassay for CRP detection using Ir-dmpq as the ECL labeling agent under the assistance of a magnet.
tuning approaches of these novel ECL labels demonstrated in this work, iridium(III) solvent complexes as novel ECL labeling agents have great prospects to develop multichannel ECL immunoassay systems.

**EXPERIMENTAL SECTION**

**Chemicals and Materials.** Iridium(III) chloride hydrate was purchased from UIV Chem (Shanghai, China). 2-Phenylpyridine (ppy), 2-(2,4-difluorophenyl)pyridine (dfppy), and 2-(3,5-dimethylphenyl)quinoline (dmpq) were obtained from commercially available sources. 2-Ethoxyethanol and acetonitrile were purchased from Sinopharm Chemical Reagent Co. Ltd. The ProCell solution (Roche Diagnostics) is used as an ECL buffer containing n-tripropylamine (TPA), in this work. Magnetic beads with the NHS group were obtained from Beaveringo Inc. (Suzhou, China) for sandwich immunoassay studies. C-reactive protein (CRP, antigen) and the corresponding antibodies were all purchased from Cusabio (Wuhan, China).

**Methods and Apparatus.** NMR spectra were recorded on a Bruker-400 magnetic resonance spectrophotometer. The solvent signals were used for 1H NMR (δ (CDCl₃) = 7.26 ppm, δ (DMSO-d₆) = 2.50 ppm, and δ (CD₃CN) = 1.94 ppm); and 13C NMR (δ (CDCl₃) = 77.16 ppm, δ (DMSO-d₆) = 39.52 ppm, and δ (CD₃CN) = 118.26 ppm) as the internal standard. TOF-MS was performed on an Agilent TOF spectrometer. A three-electrode setup was used for ECL experiments. PL quantum efficiencies (Φ) were measured in dilute acetonitrile solutions (40 μM) and compared to fac-Ir(ppy)₃ as the standard emitter according to the equation: \[ \Phi = \Phi_{ref} \left( \frac{I_s}{I_m} \right) \left( \frac{A_{ref}}{A_s} \right) \], where \( A \) is the absorbance at the excitation wavelength, \( I \) is the integrated intensity of the luminescence, and \( \Phi \) is the PL quantum efficiency. The subscripts \( s \) and \( ref \) refer to the sample and standard, respectively. A glassy carbon electrode (electrode area: 7.065 mm²) is used as the working electrode. Meanwhile, Pt wire and Ag/AgCl in saturated potassium chloride aqueous solution are the auxiliary electrode and the reference electrode, respectively. Before each experiment, the working electrode is all carefully polished with alumina (0.05 μm) and sonicated in water and ethanol in sequence. The ECL signals are all recorded on an MPI-III ECL detector (Xi’an Remax Electronics, China) equipped with a photomultiplier tube (model: R9880U-20, Hamamatsu, Japan).

**Synthesis and Characterization.** Two major steps are involved in the synthesis of iridium(III) solvent complexes in this work. First, iridium(III) chloride hydrate (1 molar equiv) and the corresponding main ligand (2 molar equiv) in the mixture solutions of 2-ethoxyethanol/water (v/v = 3:1) are refluxed for 24 h under argon atmosphere. The formed precipitate is filtered and washed with deionized water, methanol, and n-hexane in sequence, and then dried over vacuum to afford the corresponding dichloro-bridged iridium(III) dimer. Next, the dichloro-bridged iridium(III) dimer (1 molar equiv) and AgBF₄ (2 molar equiv) in acetonitrile solution are refluxed for 12 h in the dark under an argon atmosphere. Accordingly, chlorine in the dichloro-bridged iridium(III) dimer is replaced by acetonitrile under the assistance of AgBF₄ and the iridium(III) solvent complex with the corresponding main ligand is finally obtained after purification by silica gel column chromatography using acetonitrile as an eluent. Note that chiral isomers may be formed in the synthesis.

Ir-ppy (yield: 90%). 1H NMR (CD₃CN, 400 MHz), δ ppm: 9.08 (m, 1H), 8.09–8.02 (m, 2H), 7.67–7.65 (dd, J = 8, 1.2 Hz, 1H), 7.47–7.43 (m, 1H), 6.91–6.87 (td, J = 7.6, 1.2 Hz, 1H), 6.76–6.72 (td, J = 7.6, 1.2 Hz, 1H), 6.04 (dd, J = 7.6, 0.8 Hz, 1H), 1.96 (s, 3H). 13C NMR (CD₃CN, 101 Hz), δ ppm: 167.98, 151.91, 145.54, 144.69, 139.94, 132.10, 130.56, 125.20, 124.50, 123.61, 120.69, 118.34, 1.35. TOF-MS: [M – BF₄ – 2MeCN]⁺ m/z calculated. 501.0943, experimental. 501.0930.

Ir-dfppy (yield: 86%). 1H NMR (CD₃CN, 400 MHz), δ ppm: 9.08 (d, J = 2 Hz, 1H), 8.31 (d, J = 8.4 Hz, 1H), 7.98 (t, J = 8 Hz, 1H), 7.50–7.45 (m, 1H), 6.48–6.41 (m, 1H), 5.51 (dd, J = 8.4, 2.4 Hz, 1H), 2.42 (s, 3H). 13C NMR (CD₃CN, 101 Hz), δ ppm: 163.87, 163.80, 151.46, 147.07, 139.42, 123.79, 123.49, 123.30, 119.94, 114.00, 99.26, 3.85. TOF-MS: [M-BF₄]⁺ m/z, calculated 655.1097, experimental 655.1072.
Ir-dmpq (yield: 80%). $^1$H NMR (DMSO-$d_6$, 400 MHz), $\delta$ ppm: 8.60 (d, $J = 8.8$ Hz, 1H), 8.46 (d, $J = 8.8$ Hz, 1H), 8.07 (d, $J = 8$ Hz, 1H), 8.00 (d, $J = 8.8$ Hz, 1H), 7.82 (s, 1H), 7.72 (m, 1H), 7.62 (m, 1H), 6.44 (s, 1H), 2.30 (s, 3H), 2.08 (s, 3H), 1.08 (s, 3H). $^{13}$C NMR (DMSO-$d_6$, 101 Hz), $\delta$ ppm: 169.58, 147.98, 147.70, 145.45, 139.15, 137.07, 131.42, 131.07, 130.68, 128.51, 126.56, 126.47, 125.60, 125.00, 118.14, 118.00, 22.09, 20.44, 1.19. TOF-MS: [M − BF$_4$ − MeCN]$^+$ m/z, calculated 698.2147, experimental 698.2123.

**Labeling BSA with the Iridium(III) Solvent Complex.** Based on the former reported studies about the ratio of histidine to Ir-ppy at the luminescence enhancement saturation level 8, the molar ratio of the iridium(III) solvent complex to BSA was finally set to 10:1 to fully complete the labeling reaction in this work. In detail, an aliquot of 1 mM iridium(III) solvent complex in acetonitrile solution was diluted to 1 $\mu$M aqueous solution using PBS (pH = 7.4). Then, the PBS solution of the iridium(III) solvent complex and BSA with a molar ratio of 10:1 were incubated for 3 h at room temperature. After dialysis, the labeled BSA was finally obtained. The final concentration of the labeled BSA was determined by the bicinchoninic acid (BCA) assay.

**Labeling CRP Antibody with the Agent of Ir-dmpq.** This labeling method is similar to the aforementioned protocol of BSA labeling, just replacing BSA with CRP antibody in respective places.

**Sandwich Immunoassay.** The CRP antibody was first labeled on the commercial magnetic beads according to the protocols shown in the user manual of BeaverBeads Mag NHS (http://www.beaverbio.com/product/list/420.html). For sandwich immunoassay, 0.12 mg/mL of magnetic beads with the CRP antibody (the concentration of the CRP antibody on the commercial magnetic beads is ca. 3.6 $\mu$g/mL accordingly), a series of different concentrations of antigen (CRP), and 3.6 $\mu$g/mL of Ir-dmpq labeled antibody were incubated in 500 $\mu$L of PBS for 2 h at room temperature. After washing with PBS under the assistance of a magnet, the formed sandwich complexes were carefully transferred to the ECL measurement cell.

**ASSOCIATED CONTENT**

1. Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b04159.

NMR and mass spectra of iridium(III) solvent complexes and the potential studies about BSA labeled by different labels (PDF)

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