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Ascertaining Free Histidine from Mixtures with Histidine-Containing Proteins Using Time-Resolved Photoluminescence Spectroscopy

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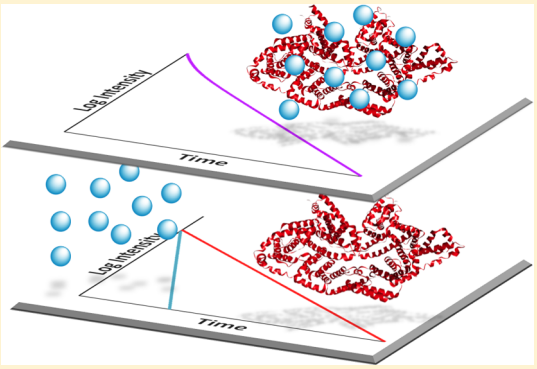
Supporting Information

ABSTRACT: The use of photoluminescent probes for differentiating free amino acids from biomolecules containing the same amino acids is challenging. Photoluminescent probes generally present similar emission spectra when in the presence of either free-amino acids or protein containing those same amino acids. Probes based on cyclometalated iridium(III) complexes $Ir(L)_2(sol)_2$ (where L is 2-phenylpyridine, 2-(2,4-difluorophenyl)pyridine, or benzo[*h*]quinolone, and sol is a solvent molecule) present long-lived emission when bound to histidine. This emission is tuned by the microenvironment around the complex and therefore its lifetime is different for free histidine (487 ns) than from histidine-containing proteins such as bovine serum albumin (average lifetime > 700 ns). As a proof-of-concept we demonstrate that free histidine can be discerned from a mixture with histidine-containing proteins by using time-resolved photoluminescence decays. In the presence of multiple sources of histidine, iridium(III) probes display a multiexponential decay, which can be fitted by nonlinear least-squares methods to separate the different components. Because the pre-exponential factor of the 487 ns lifetime is proportional to the concentration of free histidine, we can use it to assess the amount of free histidine in solution even in the presence of proteins such as bovine serum albumin. We also show that iridium(III) probes displaying different photoluminescence maxima can be produced by modifying the ancillary ligands of the metal complex.



The development of probes for the detection of histidine has been an intensive area of research in the past few years.[1−8] Interestingly, although both histidine and histidine-rich proteins are important molecules in medical diagnosis, they provide different information about diseases. Atypical levels of proteins that are rich in histidine are a sign for diseases such as AIDS,[9,10] decreased heart function,[11] familial thrombophilia,[12] advanced liver cirrhosis,[13] and malaria,[14,15] among others. On the other hand, abnormal levels of free histidine are associated with other diseases, such as mental disorders,[16] rheumatoid arthritis,[17] Alzheimer's disease,[18] and Scombroid poisoning.[19] Discrimination of histidine from histidine-containing proteins generally involves some kind of separation process followed by analysis; however, to the best of our knowledge, there are no probes reported so far capable of discerning free histidine directly from a mixture with proteins.

Transition metal complexes with low spin d^6 electronic configurations have demonstrated to have outstanding photoluminescence properties.[20−23] Among this group, iridium complexes have been widely explored as dyes for chemosensors,[24] cell staining[6,20] and biolabeling.[25] Our research group and others have explored the benefits of using iridium-based probes to detect certain amino acids.[23,25−31] Specifically for histidine, iridium probes take advantage of the coordination-based attachment of the electron pair of imidazole groups to the iridium metal center. The coordination of histidine to the iridium center produces an intense increase in photoluminescence, which has been capitalized for histidine sensing.[6,20,27,32]

As we mentioned before, the challenge of histidine probes is to distinguish the photoluminescence coming from the probe bound to histidine from the photoluminescence coming from the probe bound to other biomolecules containing histidine. Interestingly, we noticed that the photoluminescence lifetime of iridium complexes bound to histidine or histidine-containing molecules is markedly different. This is in part due to the sensitivity of the photoluminescence lifetime of iridium probes to the environment polarity, presence of oxygen, and coordination. In this research we use time-resolved photoluminescence spectroscopy in combination with long-lived iridium probes as a proof-of-concept to discriminate between free histidine and histidine-containing proteins. Specifically, we present how time-resolved decays can be analyzed to provide quantification of free histidine in solutions holding histidine-containing proteins such as bovine serum albumin (BSA), insulin, and lysozyme.

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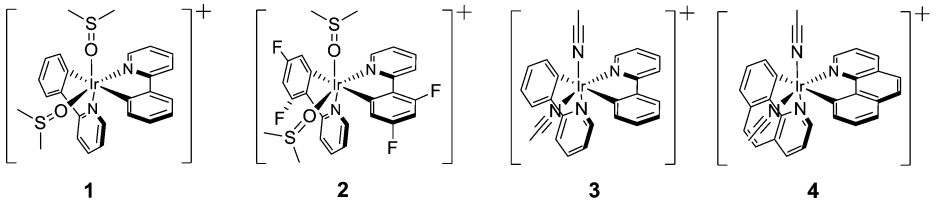
Scheme 1. Chemical Structure of $[Ir(ppy)_2(DMSO)_2]^+$ (1), $[Ir(dfppy)_2(DMSO)_2]^+$ (2), $[Ir(ppy)_2(ACN)_2]^+$ (3), and $[Ir(bq)_2(ACN)_2]^+$ (4) (ppy = 2-Phenylpyridine, fdppy = 2-(2,4-Difluorophenyl)pyridine, bq = Benzo[*h*]quinoline, ACN = acetonitrile, DMSO = Dimethyl Sulfoxide)



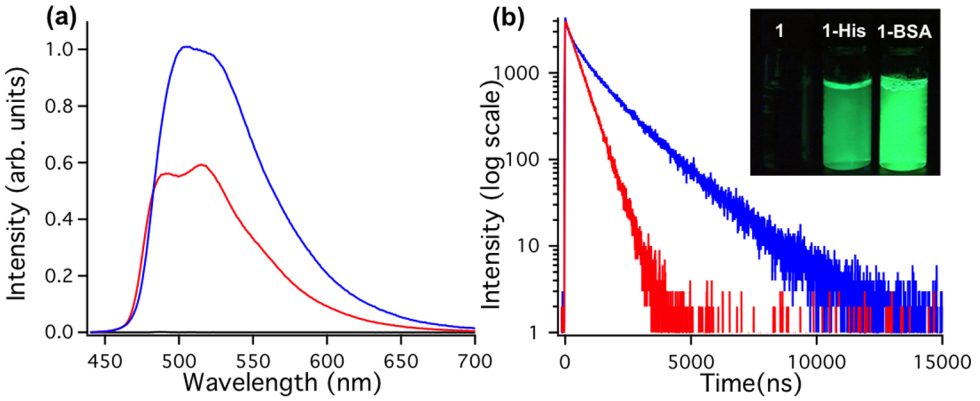


Figure 1. (a) Steady-state photoluminescence spectra of 10 μM probe **1** in PBS buffer in the absence (black line) and presence of 10 μM histidine (red line) or 10 μM BSA (blue line). (b) Decay profiles of 10 μM probe **1** in PBS buffer in the presence of 10 μM histidine (red line) or 10 μM BSA (blue line). Inset: photoluminescence photo of corresponding samples.

In this work, four histidine-responsive cyclometalated iridium(III) complexes $Ir(L)_2(sol)_2$ (Scheme 1) were synthesized using a two-step reaction (where L is 2-phenylpyridine, 2-(2,4-difluorophenyl)pyridine, or benzo[*h*]quinolone and sol is a solvent molecule). The photoluminescence response of probe **1** and **3** with histidine and histidine-containing proteins has been previously reported.[20,27] The photoluminescence spectra of these two complexes are very similar, although also very weak in the absence of histidine. When histidine is added to these complexes, they display a dramatic increase in photoluminescence. Changing the ligand from acetonitrile (ACN) to dimethyl sulfoxide (DMSO) improves the response time and the photoluminescence change of the complex. Probes **2** and **4** were also synthesized to display similar light switching behavior but with different emission wavelengths. The steady-state photoluminescence increase of these probes can be quantitatively associated with the amount of histidine, but it cannot differentiate between free and protein-bound histidine. We will demonstrate how this type of probe can be used in combination with time-resolved photoluminescence spectroscopy to quantify the amount of free histidine even in a medium with histidine-containing proteins.

In a time-resolved photoluminescence experiment, the photoluminescent molecule is excited with a short laser pulse populating the excited state. The emission from an ensemble of excited molecules in solution typically decays with a lifetime that is given according to[33]

$$I(t) = Ae^{-t/\tau} \tag{1}$$

where $I(t)$ is the time-dependent emission intensity, A is the pre-exponential factor, and τ is the photoluminescence lifetime. The emission lifetime and pre-exponential factors are determined by fitting the time decay curve to eq 1. The pre-exponential factor is the photoluminescence intensity of the dye immediately after of the end laser pulse, as long as the excitation pulse is much shorter than the probe photoluminescence lifetime, and the detector response is proportional to the concentration of produced excited states.[34] Under these conditions, the pre-exponential factor is proportional to the steady-state photoluminescence intensity as in eq 2:

$$I = \int_0^\infty Ae^{-t/\tau}\,dt = \tau A \tag{2}$$

where I is the steady-state intensity of the photoluminescent species. Because the steady-state photoluminescence intensity is proportional to the concentration of photoluminescent species, the species concentration is related to the pre-exponential factor by the following relation:

$$[P] = \delta\tau A \tag{3}$$

where δ is a proportionality factor. Equations 1−3 are illustrated using a simple single exponential function model. In reality, photoluminescent molecules in different environments are likely to display multiexponential decays of the form

$$I(t) = \sum_{i=1}^{n} A_i e^{-t/\tau_i} \tag{4}$$

where A_i values are the pre-exponential factors of the different lifetime components of the photoluminescent molecule. Therefore, the concentration of photoluminescent molecules with certain lifetime can be obtained by using the corresponding pre-exponential factor as in eq 5.

$$[P]_i = \delta_i\tau_i A_i \tag{5}$$

It is important to point out that the pre-exponential factors (A_i) are function of different parameters such as the spectral

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response of the detector, the concentration of the probe, the emission and absorption characteristics of each component (of the multiexponential decay), the spectral transmission properties of the filters and monochromators, and the spectral distribution of the excitation light.[34] Similarly, the proportionality factor (δ) depends on instrumental parameters such as slit size, laser intensity, collection time, optics, and the detector's response. Therefore, the relationship between the pre-exponential factor and concentration of a particular species corresponding to a particular decay component only holds if all other factors are kept constant.

Because the probes are nonemissive in aqueous solution, the photoluminescence intensity in the detection assay is actually proportional to the concentration of histidine, which coordinates to the iridium center and forms an emissive adduct. The photoluminescence response of probe **1** in the presence of histidine and BSA (which has 17 histidines in its sequence) is shown in Figure 1a. A typical "off-on" switch behavior was observed, in agreement with previous reports.[20] In phosphate buffer solution (PBS 6.7 mM, pH 7.2) of probe **1**, the addition of histidine produces a 1300 folds emission enhancement, which is larger than that from probe **3** (180 folds, Figure 1a and Supporting Information, Figure S15). The presence of BSA gives a larger increase in photoluminescence response of 2000 fold. As can be appreciated from Figure 1a, the photoluminescence spectrum of probe **1** with histidine is quite similar to the photoluminescence spectrum of **1** with BSA. The main difference is on the intensity, which is actually related to the different binding sites and environments between **1-His** and **1-BSA**; however, it is evident that the steady-state spectra cannot be used to differentiate between them, especially if both species are present in the same solution. Nonetheless, the time-resolved photoluminescence decays present marked differences (Figure 1b). Although the decay of **1-His** displays a monoexponential lifetime of 487 ns, **1-BSA** presents a multiexponential decay with average lifetime larger than 700 ns. The variation of **1-BSA** lifetimes as function of the concentration of **1** can be found in Table S2, Supporting Information. This longer lifetime is likely due to a more protected environment of the metal complexes when bound to BSA, which shields the complex from the molecular oxygen.

Although steady-state spectroscopy can certainly be used to report on the concentration of free histidine in samples containing no other biomolecules, detection of free histidine in a mixture with histidine-containing proteins such as BSA would be challenging using this technique. On the other hand, time-resolved spectroscopy produces decay traces that can be analyzed by nonlinear least-squares methods to resolve overlapping signals from **1** with free histidine or protein. To demonstrate this, we prepared a calibration curve of **1** with different concentrations of histidine (a typical experiment can be found in Figure 2a). **1-His** adducts exhibit single-exponential decays with fixed lifetime of 487 ns, independent of the concentration of **1-His**. Furthermore, the pre-exponential factor A_{487} (A_i value with fixed lifetime at 487 ns) as a function of concentration is linear up to 10 μM (Figure 2b). The saturation of the photoluminescence signal at 10 μM of histidine indicates that the reaction of **1** and histidine is basically stoichiometric, which was further confirmed by the ESI-MS (Figure S6, Supporting Information). A proportionality factor of $\delta = 3.3 \times 10^{-3}$ M s^{-1} was obtained from the data in Figure 2b (Supporting Information, Figure S1). This result makes it accessible to use the pre-exponential factor (A_{487}) for the

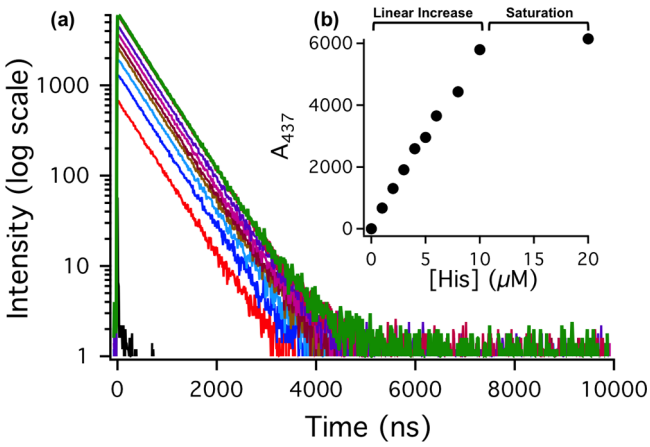

Figure 2. (a) Decay profiles of 10 μM probe **1** in PBS in the presence of different amounts of histidine (0–20 μM); (b) calculated pre-exponential factor A_{487} from the decay profiles of 10 μM solution of probe **1** as a function of histidine concentration (fixed lifetime τ at 487 ns).

selective detection of free histidine levels in the presence of background produced by histidine-containing proteins, as long as the experimental parameters used are maintained constant.

A challenging issue in the detection of histidine is its selective recognition when free histidine and histidine-containing proteins coexist in the same mixture. As a proof-of-concept of the synergy of probe **1** and time-resolved photoluminescence spectroscopy for detecting free histidine without interference from protein background, the same assay was performed in the presence of BSA (Figure 3). Different amount of histidine (0–8 μM) were added to probe **1** in PBS solution containing 0.25 μM BSA (BSA contains 17 histidines). After a period of incubation, the steady-state emission and decay profiles were obtained. The steady-state photoluminescence experiments gave a relatively small photoluminescence increase with a signal-to-background ratio (S/B) of 5.7 with 8 μM histidine. This poor S/B is due to the presence of BSA, which increases the background photoluminescence (Figure 3a). This increase in background photoluminescence causes an overestimation of the amount of His (red diamonds, Figure 3c) if steady-state photoluminescence spectroscopy is used. When the sample was probed using time-resolved spectroscopy, the result were multiexponential decays with decays signals due to **1-BSA** mixed with the single-exponential decays from **1-His** (Figure 3b). In a time-resolved experiment, the different components of a multiexponential decay can be in theory resolved if the lifetime components are different enough. The decay transients were fitted by a nonlinear least-squares routine fixing one of the component to a 487 ns lifetime (the lifetime of **1-His**). An example of how the fitting is performed is given in the Supporting Information, section 2. By using the proportionality factor δ, we calculated the concentration of histidine from the A_{487} pre-exponential factor for a solution with free histidine in the presence of BSA (Figure 3d). It is perceptible from Figure 3d that the calculated values (red diamonds) match well with the real values of histidine (blue circles), even in samples containing BSA up to a histidine concentration of 6 μM (probe concentration of 10 μM). Under the conditions of this experiment we estimate that the detection limit is ca. 1 μM (see Supporting Information, section 3 for an extended discussion on the detection limit and the photoluminescence of the system). Although not all the histidines in BSA are

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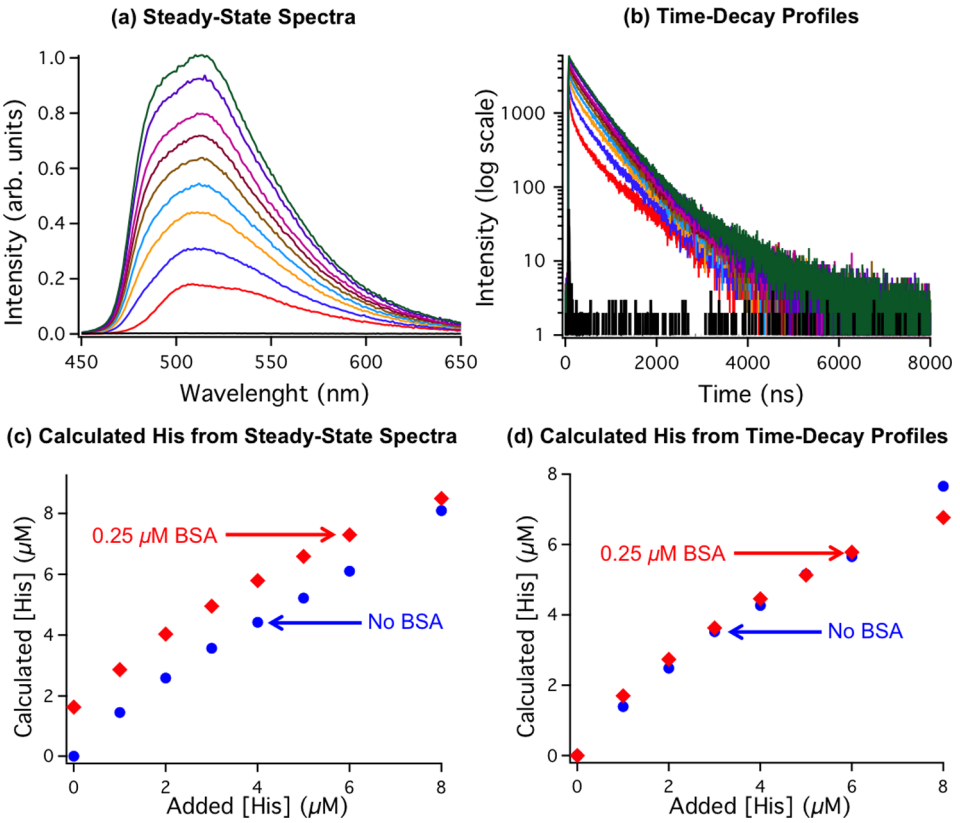

Figure 3. (a) Steady-state photoluminescence of **1** (10 μM) in the presence of different concentrations of histidine (0−8 μM) with 0.25 μM of BSA (17 histidines) in PBS. (b) Decay profiles of the samples in Figure 3a (λ_{exc} = 370 nm, λ_{em} = 510 nm). (c) Determination of the free histidine concentration from steady-state emission spectra from a solution of probe **1** in the presence (red diamonds) and absence (blue circles) of 0.25 μM BSA. (d) Determination of the free histidine concentration from the A_{487} preexponential factors obtained from a solution of probe **1** in the presence (red diamonds) and absence (black points) of 0.25 μM of BSA. Every point represents an independently prepared sample. The black curves in 3a and 3b represent 10 μM of **1** in PBS (no histidine or BSA).

expected to react with probe **1**, it is still notable being able to see an agreement for the 1 μM histidine preexponential factors (Figure 3d, blue and red markers) even in the presence of 4.3 μM protein-bound histidine (0.25 μM BSA × 17 histidines = 4.3 μM).

The correct determination of free histidine in the sample requires that all free histidines react with probe. In a system where there is an additional sink for the probe, such as BSA, there can be unreacted free histidine left at the end of the reaction. If this happens, the signal of the probe will be lower than what would be expected, and therefore the concentration of histidine will be underestimated (see 8 μM histidine concentration, Figure 3d). Therefore, the concentration of free histidine can be determined as long as there is enough probe to react with all the free histidine in the sample. Supporting Information, Figure S7, shows the same experiment as Figure 3d, but with 20 μM probe **1**. This figure shows a linear range that extends further than 10 μM histidine. Adding more probe extends the detection range because the probe is capable of reacting with all the free histidine. For a system of unknown concentrations of BSA and histidine it is recommend that the experiment is performed with at least two different concentrations of the probe. If the values obtained are consistent, then the concentration of probe is enough to react with all the free histidine; however, if the value increases with increasing probe concentration, it indicates that more probe is needed to correctly calculate the concentration of histidine in the sample. Nonetheless, given that there is enough probe to react with all the free histidine, the pre-exponential factor (A_{487}) should allow the correct calculation of free histidine even in the presence of BSA (see the correlation of the red and blue symbols in Figure 3d and Figure S7, Supporting Information). The same assay with insulin and lysozyme as background proteins were explored, which have two and one histidine in their sequence, respectively (Supporting Information, Figure S9 and S11). The results were similar to the findings with the BSA, which contains 17 histidines and is certainly a more complex environment.

It is important to point out that the success of this technique depends heavily on the ability of the nonlinear least-squares routine to separate the components (lifetimes and pre-exponential factors) which are by definition correlated in a multiexponential decay.[33] Therefore, extreme care must be taken when we analyze multiexponential decay curves with lifetimes that are similar. Also, the higher the signal-to-noise ratio, the more accurate the fit, and therefore the more accurate the determination of the different parameters. The described methodology works best when the composition of the system under study is somewhat known (e.g., the lifetimes of the probe with the protein and with the free histidine and the range of concentrations expected). Although we have demonstrated the proof-of-concept that time-resolved spectroscopy can be used in combination with photoluminescent iridium probes to detect free histidine, more experiments are still necessary to assess further advantages and limitations of this methodology.

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Because the photoluminescence emission of iridium complexes is highly dependent on the cyclometalating ligands,[21] we synthesized probes with different colors for potential multiplex detection. Probes **2**–**4** were then synthesized with modified ligands of 2-phenylpyridine. Complex **4** was synthesized with ACN and not with DMSO due to problems with purification of the DMSO product. Upon reaction with histidine, the photoluminescence emission of these complexes range from 470 to 550 nm (Figure 4). Steady-state photoluminescence and time-decay profiles experiments of probes **2**–**4** with different histidine concentrations are included in the Supporting Information (Figure S12–S20). Although the photoluminescence increase and reaction kinetics are different for the different complexes, the results suggests that all these complexes can be used in combination with time-resolved spectroscopy to detect free histidine with emission colors that range from green to orange.

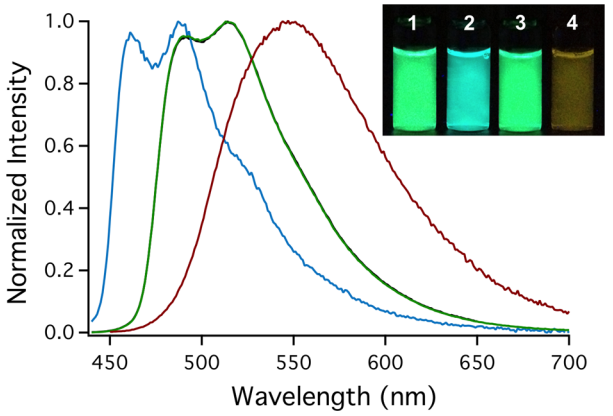

Figure 4. Normalized photoluminescence spectra of probe 1 (black line), 2 (cyan line), 3 (green line) and 4 (brown line) in PBS solution with a 2-fold excess histidine (black and green lines overlap). Inset: photographs of the photoluminescence were obtained under irradiation with UV light (365 nm).

In summary, in this manuscript it is demonstrated that pre-exponential factors derived from time-resolved experiments could be used to detect and quantify free histidine in mixtures with histidine-containing proteins. Specifically, we demonstrated the potential application of complexes such as $[Ir(ppy)_2(dmso)_2]^+$ as probes for detection of free histidine molecules in the presence of histidine-rich protein background. The pre-exponential factors obtained by using non-linear least-squares fitting routines allowed us to separate the contribution of the photoluminescence from the probe bound to free histidine and the contribution of the photoluminescence from the probe bound to histidine in the protein. The success of this technique is based on the different lifetimes presented by the probes with histidine or with proteins, which can be further applied to the detection of other free amino acids.

EXPERIMENTAL SECTION

Synthesis of 1. This compound was synthesized on the basis of literature methods.[6] The mixture of $IrCl_3$ (0.30 g) and 2-phenylpyridine (0.34 g) in solution of 2-ethoxyethanol and water (3:1, v/v) was refluxed for 24 h under nitrogen. After the mixture was cooled to room temperature, the yellow precipitate was filtered out to give crude cyclometalated iridium(III) chloro-bridged dimer. Then, a solution of cyclometalated iridium(III) chloro-bridged dimer (0.1 g) and silver nitrate (0.15 g) in 20 mL of DMSO was stirred at 100 °C under nitrogen for 4 h. When room temperature was reached, potassium hexafluorophosphate (0.15 g) was added to the dark-green solution. The suspension was kept stirring for another 15 min and filtered out. The solvent was then removed under vacuum and the crude product was purified by silica gel chromatography with an acetone/dichloromethane mixture (1:15, v/v) as eluent in a 70% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.82 (d, J = 5.6, 1H), 9.55 (d, J = 5.6, 1H), 8.27 (d, J = 8.0, 1H), 8.19 (d, J = 8.0, 1H), 8.10 (t, J = 8.0, 1H), 8.02 (t, J = 8.0, 1H), 7.80 (d, J = 8.0, 1H), 7.74 (d, J = 8.0, 1H), 7.58 (t, J = 6.8, 1H), 7.46 (t, J = 6.8, 1H), 6.91 (t, J = 7.2, 1H), 6.85 (t, J = 7.2, 1H), 6.77 (t, J = 7.2, 1H), 6.70 (t, J = 7.2, 1H), 6.26 (d, J = 7.6, 1H), 5.68 (d, J = 7.6, 1H). ESI-MS: m/e 657.2 (M − PF_6^-).

Synthesis of 2. The synthetic route is similar to the approach for **1** but only using 2-(2,4-difluorophenyl)pyridine in the first step. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 9.79 (d, J = 6.0, 1H), 9.57 (d, J = 6.0, 1H), 8.31 (d, J = 8.4, 1H), 8.26 (d, J = 8.4, 1H), 8.21 (t, J = 8.0, 1H), 8.12 (t, J = 8.0, 1H), 7.67 (t, J = 6.8, 1H), 7.58 (t, J = 6.8, 1H), 6.82 (m, 2H), 5.74 (d, J = 8.4, 1H), 5.08 (d, J = 8.4, 1H). ESI-MS: m/e 729.2 (M − PF_6^-).

Synthesis of 3. The synthetic route is similar to the approach for **1** in the first step. Acetonitrile was used as reaction solvent in the second step. ^{1}H NMR (400 MHz, acetonitrile-d_3): δ (ppm) 9.09 (d, J = 6.0, 2H), 8.05 (m, 4H), 7.65 (d, J = 7.6, 2H), 7.46 (m, 2H), 6.88 (t, J = 7.2, 2H), 6.74 (t, J = 7.6, 2H), 6.06 (d, J = 7.6, 2H), 1.96 (s, 6H). ESI-MS: m/e 583.3 (M − PF_6^-).

Synthesis of 4. The synthetic route is similar to the approach for **1**, but using benzo[h]quinolone as ligand in the first step and acetonitrile as reaction solvent in the second step. ^{1}H NMR (400 MHz, acetonitrile-d_3);: δ (ppm) 9.48 (d, J = 5.6, 2H), 8.60 (d, J = 8.0, 2H), 7.88 (m, 2H), 7.82 (s, 4H), 7.34 (d, J = 7.6, 2H), 6.94 (t, J = 7.6, 2H), 6.02 (d, J = 7.2, 2H), 1.96 (s, 6H). ESI-MS: m/e 631.3 (M − PF_6^-).

ASSOCIATED CONTENT

Supporting Information

Spectroscopic methods, general procedure to determine the proportionality factor, table of time-decay titration data, ESI-mass spectra extended discussion of the system photoluminescence, graphs of the determination of the free histidine concentration and calculated A_{613}, A_{487}, and A_{567} values, and time-decay profiles. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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