

A Highly Selective Luminescent Switch-On Probe for Histidine/Histidine-Rich Proteins and Its Application in Protein Staining**

Dik-Lung Ma, Wing-Leung Wong, Wai-Hong Chung, Fung-Yi Chan, Pui-Kin So, Tat-Shing Lai, Zhong-Yuan Zhou, Yun-Chung Leung, and Kwok-Yin Wong*

The luminescence sensing of histidine and histidine-rich proteins plays a pivotal role in biochemistry and molecular biology, in particular when both temporal and spatial resolution are required. An abnormal level of histidine-rich proteins is an indicator for many diseases, such as advanced liver cirrhosis,^[1a,b] AIDS,^[1c] renal disease,^[1c] asthma,^[1c] pulmonary disorders,^[1d] thrombotic disorders,^[1e,f] and malaria.^[1g] Some analyses of histidine and histidine-rich proteins have been developed in conjunction with immunoassay^[2a–d] and colorimetric detection methods.^[2e] The most commonly used method for the detection of histidine and histidine-rich proteins in biological samples is chromatography, which is usually performed through the combination of an effective separation technique, such as thin-layer chromatography, gas chromatography, or HPLC, followed by UV/Vis or fluorescence spectroscopy.^[2f–h] The use of high-performance capillary electrophoresis with a derivation reagent has also been reported.^[2i] However, the aforementioned methods are generally tedious, laborious, and, most importantly, expensive for routine detection in a biochemistry laboratory.

Although numerous studies have dealt with the detection of histidine or histidine-rich proteins, studies on the use of luminescent probes for this purpose remain sparse.^[3] Notable examples include research by Fabbrizzi and co-workers, who developed competitive noncovalent fluorescence turn-on probes for histidine in the form of dizinc(II) or dicopper(II) macrocyclic complexes, which recognize histidine through the formation of an imidazolate bridge between the two dizinc(II) or dicopper(II) centers;^[3a] however, the resulting noncovalent ensemble may be less stable than a covalently linking sensory

system, and the complexity of the synthetic process makes it difficult to implement in a convenient manner.

Photoluminescent iridium(III) complexes have emerged as a topical area of interest in inorganic photochemistry^[4] and phosphorescent materials for optoelectronic^[5] and luminescence signaling applications.^[6] Significant changes in the photophysical behavior and emission properties of iridium(III) complexes may be induced by the presence of biomolecules. Luminescent transition-metal complexes for protein staining, such as the luminescent ruthenium complex known as SYPRO Ruby dye, have been reported previously.^[7] However, despite its high sensitivity and its broad dynamic range, the use of SYPRO Ruby dye is limited, as it is sold only as a formulated solution; therefore, it is not possible to optimize the dye for a particular electrophoresis protocol and protein. In this context, the luminescent cyclometalated iridium(III) solvent complex $[\text{Ir}(\text{ppy})_2(\text{solv})_2]^+$ (**1**; ppy = 2-phenylpyridine, solv = H_2O or CH_3CN) has received particular attention for the following reasons: 1) $[\text{Ir}(\text{ppy})_2(\text{OH}_2)_2]^+$, which contains weakly bound solvent ligands, may bind covalently to amino acids/proteins through a ligand substitution reaction with the OH_2 ligand; 2) an intriguing solvent/media dependence of the emission properties of $[\text{Ir}(\text{ppy})_2(\text{OH}_2)_2]^+$ has been observed; 3) $[\text{Ir}(\text{ppy})_2(\text{OH}_2)_2]^+$ can be synthesized conveniently and rapidly; 4) the use of organic solvents is not required for the optimal sensing of amino acids/proteins with $[\text{Ir}(\text{ppy})_2(\text{OH}_2)_2]^+$, and the iridium complex is readily soluble and stable in aqueous staining solutions. In this study, $[\text{Ir}(\text{ppy})_3]$ (**2**) was also prepared for comparative studies, as its binding with proteins was expected to be largely hydrophobic in nature. Herein, we describe the luminescent switch-on probe $[\text{Ir}(\text{ppy})_2(\text{solv})_2]^+$ (**1**) for histidine/histidine-rich proteins and demonstrate its utility in protein staining.

The Ir^{III} complexes **1**- CF_3SO_3 and **2** (Figure 1a) were prepared according to a previously reported method.^[8,9] The structure of **1**- ClO_4 was established by X-ray crystallography and is depicted in Figure 1b, and the crystal-packing diagrams are given in the Supporting Information. The metal–ligand bonding parameters for **1**- ClO_4 are comparable to those reported previously for cyclometalated iridium(III) complexes.^[10]

The complex **1**- CF_3SO_3 (50 μM) is weakly emissive in phosphate buffered saline (PBS). In the presence of histidine (His), **1**- CF_3SO_3 exhibits an intense emission at $\lambda_{\text{max}} = 505 \text{ nm}$, the intensity of which reaches saturation level at $[\text{His}]/[\text{Ir}] \geq 4$ (Figure 2a). A plot of I/I_0 versus $[\text{His}]/[\text{1-CF}_3\text{SO}_3]$ (I and I_0 are emission intensities with and without His) shows an up-to-180-fold intensity enhancement at ratios $[\text{His}]/[\text{1-CF}_3\text{SO}_3] \geq 4:1$. The luminescence response of **1**- CF_3SO_3 to various other

[*] Dr. D.-L. Ma, Dr. W.-L. Wong, W.-H. Chung, F.-Y. Chan, P.-K. So, Dr. T.-S. Lai, Z.-Y. Zhou, Prof. Y.-C. Leung, Prof. K.-Y. Wong
Department of Applied Biology and Chemical Technology,
Central Laboratory of the Institute of Molecular Technology for Drug
Discovery and Synthesis,
The Hong Kong Polytechnic University
Hung Hom, Kowloon, Hong Kong (China)
Fax: (+1) 852-2364-9932
E-mail: bcedmond@polyu.edu.hk
bckywong@inet.polyu.edu.hk

[**] This research was supported by The Hong Kong Polytechnic University and the Area of Excellence Fund of the University Grants Committee (AoE/P-10/01). D.L.M. and W.L.W. acknowledge the award of postdoctoral fellowships administered by the Research Committee of The Hong Kong Polytechnic University. We are grateful to Prof. Chi-Ming Che for supporting this research and for his invaluable suggestions.

Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

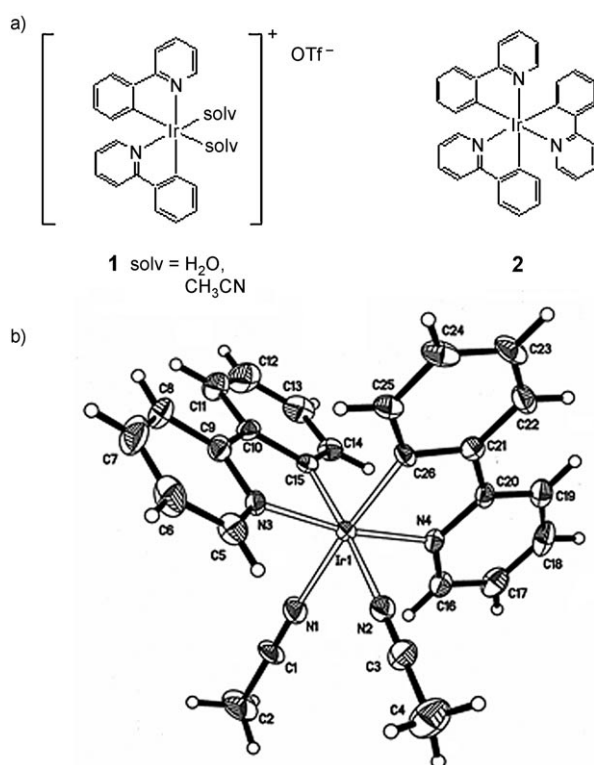


Figure 1. a) Structures of **1**-CF₃SO₃ and **2**. b) X-ray crystal structure of the cation of **1** (from crystals of the ClO₄⁻ salt). OTf = trifluoromethanesulfonyl.

natural amino acids was also investigated. No significant changes in the emission of **1**-CF₃SO₃ were observed upon the addition of natural amino acids (Figure 2b). Competition experiments were carried out in the presence of His, as well as in a mixture containing His and 10 equivalents of another natural amino acid. No significant difference in luminescence intensity from that observed in the presence of only His was found in these experiments. This result indicates that **1**-CF₃SO₃ displays high selectivity for His.

We analyzed the binding of **1** with histidine by electrospray-ionization positive-ion mass spectrometry. After the incubation of histidine with **1** (*m/z* 501.1) for 5 h at 20 °C, a new major peak was observed (Figure 3b). This peak, centered at *m/z* 656.1, corresponds to the covalent attachment of one histidine molecule (*m/z* 155) to **1**. No mass change was recorded for **1** upon incubation with alanine, which does not contain an imidazole functional group, under similar reaction conditions (Figure 3c). These findings, together with the results for the luminescence response of **1**-CF₃SO₃ to various natural amino acids, suggests that the luminescent cyclometalated iridium(III) solvent complex **1**-CF₃SO₃ may specifically recognize histidine through covalent attachment to the imidazole moiety of histidine rather than to the carboxylate group of other natural amino acids.

The luminescence response of **1**-CF₃SO₃ to proteins with a relatively high abundance of histidine residues, such as bovine serum albumin (BSA), was also investigated. In the presence of BSA, **1**-CF₃SO₃ exhibits an intense emission at $\lambda_{\text{max}} = 505$ nm, the intensity of which reaches saturation level at

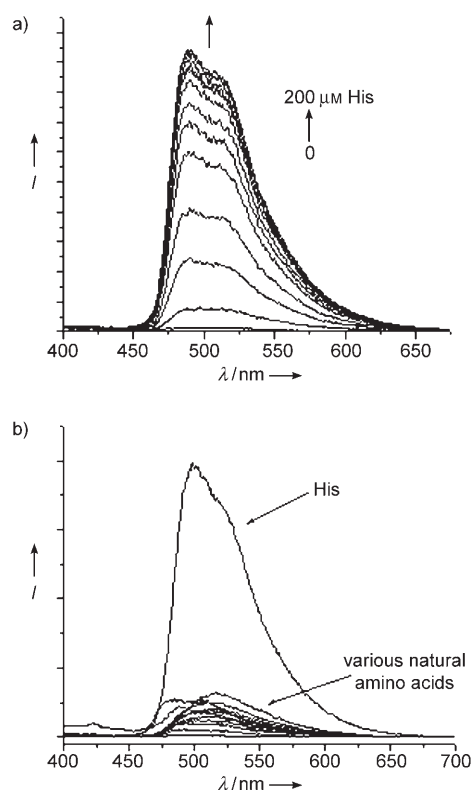


Figure 2. a) Emission spectra of **1**-CF₃SO₃ (50 μM) in PBS buffer with increasing concentrations of [His]/[Ir] (0–4.0) at 20.0 °C. b) Emission spectra of **1**-CF₃SO₃ (50 μM) in PBS buffer with various natural amino acids (200 μM) at 20.0 °C.

[BSA]/[Ir] ≥ 100 (Figure 4). A plot of I/I_0 versus [BSA]/[**1**-CF₃SO₃] (I and I_0 are emission intensities with and without BSA) shows an up-to-800-fold intensity enhancement at ratios [BSA]/[**1**] ≥ 100:1. No enhancement in emission intensity was recorded for **2** in a similar emission-titration study with BSA.

Common spectrophotometric methods, such as the Bradford,^[11] bicinchoninic acid (BCA),^[12] and Lowry^[13] methods, have been used extensively for protein quantification. However, these in-solution methods are restricted by the presence of interfering substances, including metal ions, sodium dodecyl sulfate (SDS), and ethylenediaminetetraacetic acid (EDTA). Fluorometric protein assays, which are generally more sensitive, have been reported.^[14] The luminescence response of **1**-CF₃SO₃ to various nonprotein substances, such as inorganic salts (sodium chloride, sodium acetate, sodium phosphate, cobalt chloride, zinc chloride, calcium chloride), the chelating agent EDTA, and detergents (SDS, Triton X-100) was also examined. All tests were performed with BSA (5 mM) mixed with **1**-CF₃SO₃ (50 μM) in the presence of an excess amount of the foreign substance. A less than 5% change in luminescence intensity was observed upon the addition of the foreign substance. These results revealed that the response of **1**-CF₃SO₃ to BSA was not affected by nonprotein substances present in excess.

Solid-phase protein assays and gel electrophoresis provide good alternatives to avoid interfering substances.^[15] With the protein immobilized on a solid surface, such as a nitro-

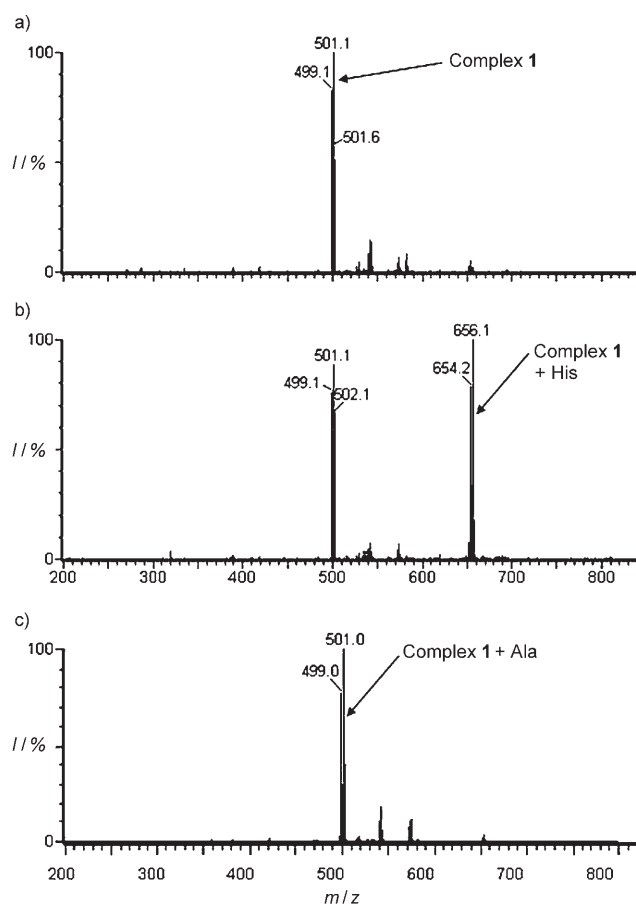


Figure 3. Electrospray-ionization positive-ion mass spectra of a) complex **1**, b) reaction mixture of complex **1** and histidine, and c) reaction mixture of complex **1** and alanine.

cellulose membrane, or the protein electrophoresized on a gel, interfering substances can be washed away. Thus, we examined the staining of a series of proteins in sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE) with the luminescent iridium(III) complex **1**-CF₃SO₃. A mixture of commercially available protein markers was used for the experiments. Figure 5 shows an emissive image of a gel containing electrophoresized protein markers (total protein per lane: 0.8–400 ng) after staining with **1**-CF₃SO₃ (1.2 mg/20 mL) for 10 min. The lowest quantity of the protein mixture detected after staining with **1**-CF₃SO₃ was 1.5 ng (lane I in Figure 5).

Conventional protein-staining methods include colloidal-gold staining, colloidal-silver staining, Coomassie Blue staining, Ponceau S staining, pyrogallol red molybdate staining, India-ink staining, and double-metal-chelate (DMC) staining.^[15c,d,16–18] Many of these methods, however, involve time-consuming procedures and multiple reagents. The most frequently used and commercially available Coomassie Brilliant Blue (CBB)^[17] stain requires a long time for destaining (overnight): Staining for 30 min followed by destaining for 24 h led to a significant reduction in background staining and hence consequently improved band contrast. A destaining step was not required for **1**-CF₃SO₃,

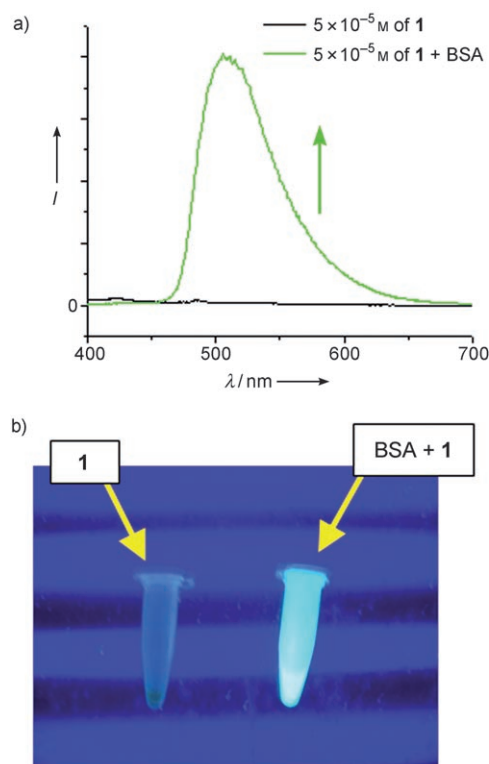


Figure 4. a) Emission spectra of **1**-CF₃SO₃ (50 μM) in PBS buffer with [BSA]/[Ir]=0 and [BSA]/[Ir]=100 at 20.0 °C. b) Photographs of **1**-CF₃SO₃ in the absence and in the presence of BSA.

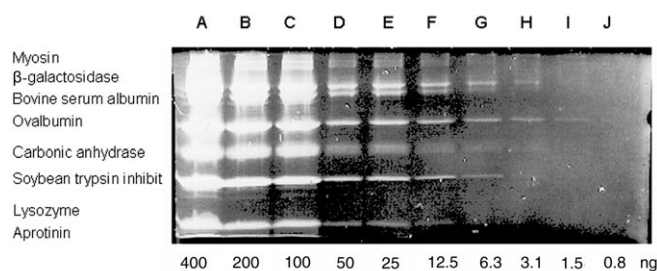


Figure 5. Emissive SDS-PAGE analysis of a commercially available mixture of protein markers with **1**-CF₃SO₃ (1.2 mg/20 mL) as the detection stain (staining time: (10 ± 1) min).

and there was no increase in signal intensity after destaining for 24 h. Methods based on commercially available SYPRO Ruby dyes^[7] require 7% acetic acid in the staining solution. The presence of acetic acid is problematic during electroblotting, electroelution, and measurements of enzyme activity. In contrast, **1**-CF₃SO₃ does not require the use of organic solvents for optimal staining of proteins and is readily soluble and stable in aqueous staining solutions. Finally, the staining procedure with **1**-CF₃SO₃ is rapid and simple, requires minimal labor, and enables the detection of as little as 1.5 ng of a poly(amino acid) per band. This sensitivity is in many cases equal to that of silver staining methods,^[17] however, the procedure with **1**-CF₃SO₃ is much less hazardous and much less expensive.

To obtain more information on the linear dynamic range of $1\text{-CF}_3\text{SO}_3$, we performed an experiment with $1\text{-CF}_3\text{SO}_3$ with a total protein amount ranging from 1200 to 0.01 ng. The linear dynamic range of $1\text{-CF}_3\text{SO}_3$ staining extends from 0.1 to 1000 ng (R value = 0.9998). Extended linear response is a property of the utmost importance for comparisons of protein expression in analyses with 2-D gels and peptide-mass fingerprinting (PMF).

The complex $1\text{-CF}_3\text{SO}_3$ can also be used to distinguish histidine-rich biomolecules, such as histidine-rich proteins, through a detectable luminescence response in a buffered solution or SDS-PAGE gel. To minimize the response that originates from other amino acid sequences, the isoelectric point (pI), the protein structure, and the presence of certain side chains, β -lactamase tagged with six His residues and non-His-tagged β -lactamase were used in the present study. The His-tagged β -lactamase could be detected in a gel or in solution on the basis of a strong luminescence signal after incubation with $1\text{-CF}_3\text{SO}_3$ for 10 min. The non-His-tagged β -lactamase showed much weaker luminescence (Figure 6). This property may be useful for keeping track of histidine-rich proteins during nickel-column purification.

We next demonstrated the utility of $1\text{-CF}_3\text{SO}_3$ as a staining reagent for the detection of a mixture of proteins in a Western blot. A protein mixture (ca. 0.5 mg) was subjected to Western blot analysis followed by staining with $1\text{-CF}_3\text{SO}_3$ (1.2 mg/20 mL) for 10 min (see the Supporting Information). The

result showed that the detection of proteins with $1\text{-CF}_3\text{SO}_3$ can also be applied to Western blot analysis.

In summary, the new luminescent switch-on probe $1\text{-CF}_3\text{SO}_3$ exhibits a 180-fold increase in the intensity of photoluminescence at $\lambda_{\text{max}} = 505$ nm and features excellent selectivity for histidine over other natural amino acids. Moreover, we have developed this cyclometalated iridium(III) solvent complex, which has been used previously as a catalyst or catalyst precursor in homogeneous photoreduction, photoelectrochemical reduction, and electrochemical reduction, as a stain for proteins in SDS-PAGE gel. In terms of detection sensitivity, linear dynamic range, and staining time, $1\text{-CF}_3\text{SO}_3$ is comparable to SYPRO Ruby stains. The staining time for $1\text{-CF}_3\text{SO}_3$ is 10 min; no destaining step and no organic solvents are required. Given the fact that the emission properties of $[\text{Ir}(\text{ppy})_2(\text{solv})_2]^+$ can be modified through auxiliary ligand(s), we envision further improvement in protein detection through the rational molecular design of luminescent iridium(III) complexes.

Received: November 20, 2007

Revised: February 20, 2008

Published online: April 15, 2008

Keywords: electrophoresis · histidine · iridium · luminescent probes · protein staining

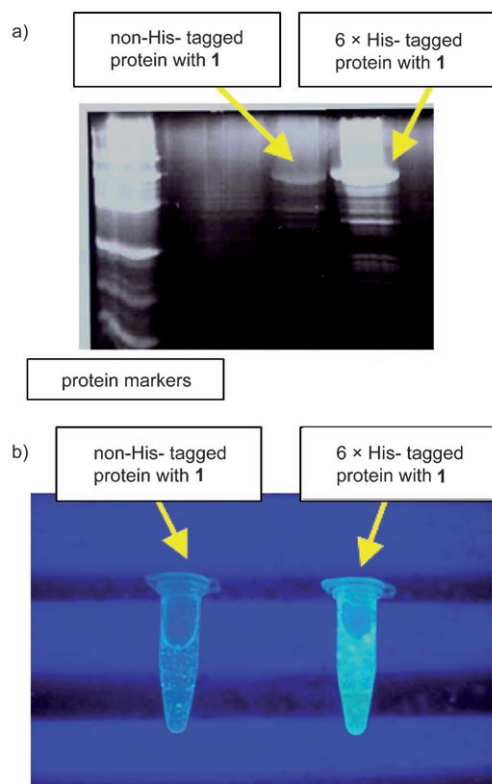


Figure 6. a) Emissive SDS-PAGE analysis of 6xHis-tagged β -lactamase and non-His-tagged β -lactamase with $1\text{-CF}_3\text{SO}_3$ (1.2 mg/20 mL) as the detection stain (staining time: (10 ± 1) min). b) Photographs of $1\text{-CF}_3\text{SO}_3$ in the presence of 6xHis-tagged β -lactamase and non-His-tagged β -lactamase.

- [1] a) H. Saito, L. T. Goodnough, J. M. Boyle, N. Heimbürger, *Am. J. Med.* **1982**, 73, 179; b) F. W. Leebeek, C. Kluft, E. A. Knot, M. P. De Matt, *J. Lab. Clin. Med.* **1989**, 113, 493; c) A. L. Jones, M. D. Hulett, C. R. Parish, *Immunol. Cell Biol.* **2005**, 83, 106; d) W. T. Morgan, *Biochem. Med. Metab. Biol.* **1986**, 36, 210; e) L. Engesser, C. Kluft, E. Briet, E. J. Brommer, *Br. J. Haematol.* **1987**, 67, 355; f) C. Kuhli, I. Scharrer, F. Koch, L. O. Hattenbach, *Am. J. Ophthalmol.* **2003**, 135, 232; g) D. J. Sullivan, Jr., I. Y. Gluzman, D. E. Goldberg, *Science* **1996**, 271, 219.
- [2] a) M. E. Parra, C. B. Evans, D. W. Taylor, *J. Clin. Microbiol.* **1991**, 29, 1629; b) C. J. N. Shiff, J. Minjas, Z. Premji, *Trans. R. Soc. Trop. Med. Hyg.* **1993**, 87, 646; c) R. F. G. Leke, R. R. Djokam, R. Mbu, R. J. Leke, J. Fogako, R. Megnekou, S. Metenou, G. Sama, Y. Zhou, T. Cadigan, M. Parra, D. W. Taylor, *J. Clin. Microbiol.* **1999**, 37, 2992; d) T. Jelinek, M. P. Grobusch, G. Harms, *J. Infect. Dis.* **2001**, 33, 752; e) D. Sahal, R. Kannan, A. Sinha, V. Babbarwal, B. G. Prakash, G. Singh, V. S. Chauhan, *Anal. Biochem.* **2002**, 308, 405; f) P. Leroy, A. Nicolas, A. Moreau, *J. Chromatogr.* **1983**, 282, 561; g) T. B. Jensen, P. D. Marely, *J. Chromatogr. B* **1995**, 670, 199; h) N. Tateda, K. Matsuhisa, K. Hasebe, N. Kitagima, T. Miura, *J. Chromatogr. B* **1998**, 718, 235; i) R. M. LaTorre, J. Saurina, S. Hernandez-Cassou, *J. Chromatogr. Sci.* **1999**, 37, 353.
- [3] a) M. A. Hortalá, L. Fabbriizzi, N. Marcotte, F. Stomeo, A. Taglietti, *J. Am. Chem. Soc.* **2003**, 125, 20; b) Y. Zhang, R.-H. Yang, F. Liu, K.-A. Li, *Anal. Chem.* **2004**, 76, 7336.
- [4] a) M. A. Baldo, S. Lamansky, P. E. Burrows, M. E. Thompson, S. R. Forrest, *Appl. Phys. Lett.* **1999**, 75, 4; b) M. Ikai, S. Tokito, Y. Sakamoto, T. Suzuki, Y. Taga, *Appl. Phys. Lett.* **2001**, 79, 156; c) A. B. Tamayo, B. D. Alleyne, P. I. Djurovich, S. Lamansky, I. Tsyba, N. N. Ho, R. Bau, M. E. Thompson, *J. Am. Chem. Soc.* **2003**, 125, 7377; d) P. Coppo, E. A. Plummer, L. De Cola, *Chem. Commun.* **2004**, 1774; e) A. Tsuboyama, T. Takiguchi, S. Okada, M. Osawa, M. Hoshino, K. Ueno, *Dalton Trans.* **2004**, 1115; f) M.-K. Lau, K.-M. Cheung, Q.-F. Zhang, Y. Song, W.-T. Wong,

- I. D. Williams, W.-H. Leung, *J. Organomet. Chem.* **2004**, 689, 2401.
- [5] a) M. A. Baldo, M. E. Thompson, S. R. Forrest, *Nature* **2000**, 403, 750; b) V. V. Grushin, N. Herron, D. D. LeCloux, W. J. Marshall, V. A. Petrov, Y. Wang, *Chem. Commun.* **2001**, 1494; c) F. C. Chen, Y. Yang, M. E. Thompson, J. Kido, *Appl. Phys. Lett.* **2002**, 80, 2308; d) M. K. Nazeeruddin, R. Humphry-Baker, D. Berner, S. Rivier, L. Zuppiroli, M. Graetzel, *J. Am. Chem. Soc.* **2003**, 125, 8790; e) A. Beeby, S. Bettington, I. D. W. Samuel, Z. Wang, *J. Mater. Chem.* **2003**, 13, 80; f) J. D. Slinker, A. A. Gorodetsky, M. S. Lowry, J. Wang, S. Parker, R. Rohl, S. Bernhard, G. G. Malliaras, *J. Am. Chem. Soc.* **2004**, 126, 2763; g) S. Jung, Y. Kang, H.-S. Kim, Y.-H. Kim, C.-L. Lee, J.-J. Kim, S.-K. Lee, S.-K. Kwon, *Eur. J. Inorg. Chem.* **2004**, 3415; h) A. J. Sandee, C. K. Williams, N. R. Evans, J. E. Davies, C. E. Boothby, A. Kohler, R. H. Friend, A. B. Holmes, *J. Am. Chem. Soc.* **2004**, 126, 7041; i) J. I. Kim, I.-S. Shin, H. Kim, J.-K. Lee, *J. Am. Chem. Soc.* **2005**, 127, 1614.
- [6] a) M. C. DeRosa, D. J. Hodgson, G. D. Enright, B. Dawson, C. E. B. Evans, R. J. Crutchley, *J. Am. Chem. Soc.* **2004**, 126, 7619; b) K. K.-W. Lo, W.-K. Hui, C.-K. Chung, K. H.-K. Tsang, T. K.-M. Lee, C.-K. Li, J. S.-Y. Lau, D. C.-M. Ng, *Coord. Chem. Rev.* **2006**, 250, 1724; c) Q. Zhao, T. Cao, F. Li, X. Li, H. Jing, T. Yi, C. Huang, *Organometallics* **2007**, 26, 2077; d) M. Schmittel, H. Lin, *Inorg. Chem.* **2007**, 46, 9139.
- [7] a) K. Berggren, E. Chernokalskaya, T. H. Steinberg, C. Kemper, M. F. Lopez, Z. Diwu, R. P. Haugland, W. F. Patton, *Electrophoresis* **2000**, 21, 2509; b) R. P. Haugland, *Handbook of Fluorescent Probes and Research Products*, 9th ed., Molecular Probes, Leiden, **2002**.
- [8] B. Schmid, F. O. Garces, R. J. Watts, *Inorg. Chem.* **1994**, 33, 9.
- [9] K. A. King, P. J. Spellane, R. J. Watts, *J. Am. Chem. Soc.* **1985**, 107, 1431.
- [10] H.-C. Böttcher, M. Graf, H. Krüger, C. Wagner, *Inorg. Chem. Commun.* **2005**, 8, 278.
- [11] M. M. Bradford, *Anal. Biochem.* **1976**, 72, 248.
- [12] P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, D. C. Klenk, *Anal. Biochem.* **1985**, 150, 76.
- [13] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **1951**, 193, 265.
- [14] B. K. Hoefelschweiger, A. Duerkop, O. S. Wolfbeis, *Anal. Biochem.* **2005**, 344, 122.
- [15] a) H. Kuno, H. K. Kihara, *Nature* **1967**, 215, 974; b) J. B. Sheffield, D. Graff, H. P. Li, *Anal. Biochem.* **1987**, 166, 49; c) J. B. Hunter, S. M. Hunter, *Anal. Biochem.* **1987**, 164, 430; d) L. S. Minamide, J. R. Bamberg, *Anal. Biochem.* **1990**, 190, 66; e) M. J. Lim, W. F. Patton, M. F. Lopez, K. H. Spofford, N. Shojaei, D. Shepro, *Anal. Biochem.* **1997**, 245, 184; f) T. Morcol, A. Subramanian, *Anal. Biochem.* **1999**, 270, 75; g) T. Nishibu, K. Hirayasu, T. Tanaka, Y. Takeda, Y. Kobayashi, *Anal. Biochem.* **2003**, 319, 88; h) B. J. Agnew, D. Murray, W. F. Patton, *Electrophoresis* **2004**, 25, 2478.
- [16] a) K. Hancock, V. C. Tsang, *Anal. Biochem.* **1983**, 133, 157; b) M. Moeremans, M. De Raeymaeker, G. Daneels, J. De Mey, *Anal. Biochem.* **1986**, 153, 18; c) A. Kovarik, K. Hlubinova, A. Vrbenska, J. Prachar, *Folia Biol.* **1987**, 33, 253; d) P. Mitra, A. K. Pal, D. Basu, R. N. Hati, *Anal. Biochem.* **1994**, 223, 327.
- [17] V. Neuhoff, R. Stamm, H. Eibl, *Electrophoresis* **1985**, 6, 427.
- [18] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, *Anal. Chem.* **1996**, 68, 850.