ChemComm

RSC Publishing

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2013, **49**, 4271

Received 24th September 2012, Accepted 18th October 2012

DOI: 10.1039/c2cc36907a

www.rsc.org/chemcomm

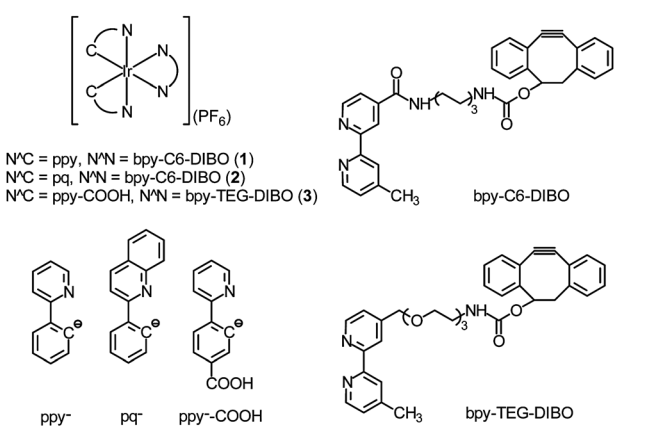
Cyclometalated iridium(III) polypyridine dibenzocyclooctyne complexes as the first phosphorescent bioorthogonal probes†‡

Kenneth Kam-Wing Lo,* Bruce Ting-Ngok Chan, Hua-Wei Liu, Kenneth Yin Zhang, Steve Po-Yam Li and Tommy Siu-Ming Tang

We report the synthesis, photophysical behavior, and biological properties of new cyclometalated iridium(III) polypyridine complexes appended with a dibenzocyclooctyne (DIBO) moiety; these complexes have been utilized as the first phosphorescent bioorthogonal probes for azide-modified biomolecules.

Bioorthogonal chemistry, pioneered by Bertozzi and co-workers, has emerged as a versatile method to image biomolecules such as glycans, lipid-modified proteins, nucleic acids, and secondary metabolites in their native environments.[1] In a typical procedure, a substrate modified with a chemical reporter is incorporated into live cells or organisms, which is then recognized by a bioorthogonal probe that carries the complementary functionality.[1] Among the chemical reporters, the azide is particularly useful due to its small size, non-native nature, and inertness toward biomolecules. Additionally, it can selectively undergo the Staudinger ligation with phosphines,[1a] copper(I)-catalyzed cycloaddition with alkynes,[2] and strain-promoted alkyne–azide cycloaddition (SPAAC) with cyclooctynes,[3] allowing the design of a wide range of probes. The SPAAC is considered as a very attractive strategy for bioimaging because it is highly efficient and selective, and the use of cytotoxic copper(I) as a catalyst is not required. The design of bioorthogonal probes to trace azide-labeled biomolecules has mainly relied on the use of affinity tags,[3] fluorescent organic dyes,[3] and luminescent quantum dots.[4] In view of their interesting emission characteristics, such as high photostability and long emission lifetimes, phosphorescent inorganic and organometallic transition metal complexes such as those of ruthenium(II), rhenium(I), and iridium(III) have emerged as promising candidates for biological labeling and cellular imaging applications; interesting organelle-specific staining capability has also been observed.[5] Despite this rapid development, to the best of our knowledge, the possibility of applying phosphorescent inorganic and organometallic transition metal complexes as bioorthogonal probes for biomolecules in their native settings has not been explored. With our on-going interest in the design of phosphorescent transition metal polypyridine complexes as biomolecular and cellular probes,[6] we envisage that the incorporation of a strained cyclooctyne moiety into these complexes will generate a new class of reagents capable of targeting different azide-labeled biomolecules for imaging and therapeutic applications.

First, we designed two phosphorescent cyclometalated iridium(III) polypyridine complexes $[Ir(N^C)_2(bpy\text{-}C6\text{-}DIBO)](PF_6)$ (bpy-C6-DIBO = 4-(*N*-(6-(3,4:7,8-dibenzocyclooctyne-5-oxycarbonylamino)hexyl)aminocarbonyl)-4′-methyl-2,2′-bipyridine; HN^C = 2-phenylpyridine Hppy (**1**), 2-phenylquinoline Hpq (**2**)) containing a DIBO moiety (Scheme 1). The DIBO unit was selected since it is nontoxic and easy to prepare, and has high reactivity toward azides.[3b] The synthesis of the diimine ligand bpy-C6-DIBO is given in ESI.‡ Treatment of dibenzocyclooctynol with 4-nitrophenyl chloroformate gave a carbonate ester, which was reacted with the amine bpy-C6-NH_2 to afford the diimine ligand bpy-C6-DIBO. Complexes **1** and **2** were prepared from the reaction of the iridium(III) dimer $[Ir_2(N^C)_4Cl_2]$ with bpy-C6-DIBO in a



Scheme 1 Structures of complexes **1–3**.

Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, People's Republic of China. E-mail: bhkenlo@cityu.edu.hk; Fax: +852 3442 0522

† This article is part of the *ChemComm* 'Emerging Investigators 2013' themed issue.

‡ Electronic supplementary information (ESI) available: Synthesis, characterization, photophysical data, kinetics studies, lipophilicity, cellular uptake efficiency, cytotoxicity, bioconjugation, microscopy images, and flow cytometry results. See DOI: 10.1039/c2cc36907a

Table 1 Photophysical data of complexes **1–3** at 298 K

Complex	Medium	λ_{em}/nm	τ_o/μs	Φ_{em}
1	CH_2Cl_2	595	0.47	0.13
	CH_3CN	607	0.25	0.033
	MeOH	610	0.091	0.020
	Glass[a]	533, 570 sh	4.71	
2	CH_2Cl_2	558, 590 sh	0.95	0.26
	CH_3CN	564, 595 sh	0.63	0.082
	MeOH	551 sh, 595	0.16	0.031
	Glass[a]	542 (max), 584, 635 sh	4.77	
3	CH_2Cl_2	512 (max), 541	3.27	0.76
	CH_3CN	509 (max), 541	3.63	0.66
	MeOH	514 sh, 553	1.78 (43%), 0.48 (57%)	0.20
	Glass[a]	501 (max), 542, 582 sh	6.61	

[a] EtOH/MeOH (4:1, v/v) at 77 K.

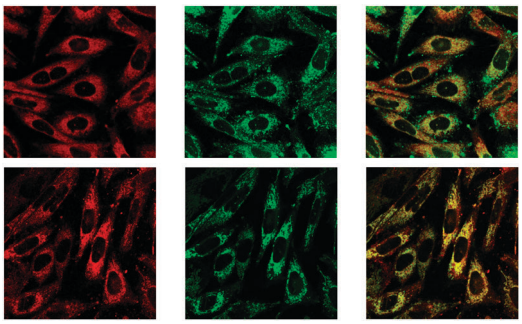


Fig. 1 Confocal microscopy images of CHO cells incubated with complexes **1** (top) and **2** (bottom), respectively (30 μM, 37 °C, 20 min) and MitoTracker (100 nM, 37 °C, 15 min). λ_{ex} = 405 nm (left) and 633 nm (middle), the overlaid images are shown on the right.

mixture of CH_2Cl_2 and MeOH, followed by anion exchange and column chromatographic purification. Both complexes were characterized by ^{1}H NMR spectroscopy, positive-ion ESI-MS, IR spectroscopy, absorption spectroscopy, and microanalysis (ESI‡).

Upon photoexcitation, complexes **1** and **2** exhibited intense and long-lived green to orange emission in fluid solutions at 298 K and in alcohol glass at 77 K (Table 1). Complex **1** displayed a broad emission band with positive solvatochromism, which is a typical property of ^{3}MLCT (dπ(Ir) → π^*(bpy-C6-DIBO)) and ^{3}LLCT (π(ppy) → π^*(bpy-C6-DIBO)) emitters.[7] In contrast, the emission band of complex **2** had structural features and the emission was longer-lived and less solvent-dependent, indicative of a ^{3}IL ($\pi \rightarrow \pi^*$) (pq) excited state.[7] Note that the incorporation of a DIBO unit through a C6-spacer arm did not have any significant effects on the photophysical properties of both complexes, as revealed by a comparison of their emission data with those of other iridium(III) ppy and pq complexes.[7c]

To investigate the reactivity of their DIBO pendants, both complexes were reacted with a model substrate benzyl azide. The resultant triazole complexes **1a** and **2a** were obtained as a mixture of regioisomers in quantitative yields (ESI‡). The kinetics for the reaction of complex **2** with benzyl azide in $(CD_3)_2CO$ at 298 K was monitored by ^{1}H NMR spectroscopy. The second-order rate constant was determined to be $4.2 \pm 0.8 \times 10^{-2}$ M^{-1} s^{-1} (ESI‡), which is similar to other DIBO–azide systems.[3b] Unfortunately, the reaction kinetics of complex **1** could not be examined as its solubility is not sufficiently high for accurate measurements by NMR. It is noteworthy that the photophysical data of complexes **1a** and **2a** are indistinguishable from those of their precursor complexes **1** and **2** (ESI‡). Next, we studied the labeling of bovine serum albumin (BSA), human serum albumin (HSA), and apotransferrin (aTf) that had been modified with azidoacetic acid (ESI‡). The conjugates were purified by size-exclusion chromatography and membrane filtration, and characterized by SDS-PAGE. As expected, all the conjugates inherited the attractive emission characteristics of complexes **1** and **2** (ESI‡).

Lipophilicity studies revealed that complex **2** had a larger log $P_{o/w}$ value (7.14) than complex **1** (5.69) (ESI‡), due to an additional fused benzene ring on the ligand pq. Thus, complex **2** was expected to be more efficiently taken up by mammalian cells. However, ICP-MS measurements using Chinese hamster ovary (CHO) cells indicated less efficient uptake for complex **2** (ESI‡). This suggests that the uptake may be favored by a smaller molecular size. Incubation of CHO cells with the complexes (30 μM, 37 °C, 20 min) resulted in very efficient uptake and subsequent localization in the perinuclear region. Costaining experiments revealed that the complexes were mostly located at mitochondria (Fig. 1), which has been reported for other transition metal complexes.[8] The cytoskeletal inhibitor colchicine, the ATPase inhibitor KNO_3, and incubation at 4 °C, respectively, did not substantially decrease the uptake efficiency, which indicated that the mode of internalization is passive diffusion (ESI‡).[5] This is also in agreement with the more efficient uptake of the smaller complex **1**. Since 1,3,4,6-tetra-*O*-acetyl-*N*-azidoacetyl-D-mannosamine (Ac_4ManNAz) is metabolically converted by CHO cells to *N*-azidoacetyl sialic acids, which are located on the cell surface,[3a,b,d] pretreatment of CHO cells with Ac_4ManNAz is expected to enhance the localization of the complexes in the plasma membrane. However, confocal microscopy images and flow cytometry revealed that the staining pattern and emission intensity of Ac_4ManNAz-treated and -untreated CHO cells were very similar (ESI‡). We have attributed this to the highly efficient internalization of both complexes by the cells, which significantly limits the labeling of azido sialic acid residues within the cell-surface glycans. ICP-MS measurements also showed that the Ac_4ManNAz pretreatment increased the iridium uptake by only *ca.* 0.05 and 0.07 fmol per average CHO cell for complexes **1** and **2**, respectively (ESI‡), which is one order of magnitude smaller than the uptake of the complexes themselves.

Taking the above into consideration, we aimed to design new iridium–DIBO complexes with lower cellular uptake efficiency. Thus, we have synthesized $[Ir(ppy\text{-}COOH)_2(bpy\text{-}TEG\text{-}DIBO)](PF_6)$ (bpy-TEG-DIBO = 4-(10-*N*-(3,4:7,8-dibenzocyclooctyne-5-oxycarbonyl)-amino-2,5,8-trioxa-decyl)-4′-methyl-2,2′-bipyridine) (**3**) with two polar ppy-COOH ligands and a triethylene glycol (TEG) linker (Scheme 1). The deprotonated form of the cyclometalating ligand in aqueous solution should limit the cellular uptake of the complex and hence facilitate the SPAAC reaction.[1c] Additionally, the TEG spacer-arm is anticipated to increase the water solubility, polarity, and biocompatibility of the complex. The synthesis of bpy-TEG-DIBO involved the reaction of bpy-TEG-OMs (4-(10-methylsulfonyloxy-2,5,8-trioxa-decyl)-4′-methyl-2,2′-bipyridine) with NaN_3, followed by the Staudinger

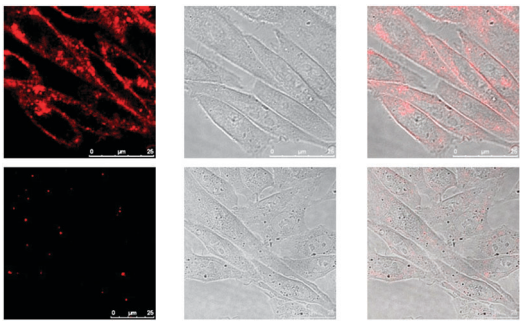


Fig. 2 Fluorescence (left), brightfield (middle), and overlaid (right) confocal microscopy images of Ac_4ManNAz-treated (top) and -untreated (bottom) CHO cells incubated with complex **3** (30 μM, 37 °C, 5 h).

reduction to an amine derivative, and a subsequent reaction with the DIBO carbonate ester (ESI‡). We reacted this diimine ligand with the iridium(III) dimer $[Ir_2(ppy\text{-}COOH)_4Cl_2]$ and obtained complex **3** after anion exchange (ESI‡). Upon irradiation, this complex exhibited intense and long-lived emission in the green to yellow region with a structured band (Table 1), the origin of which has been assigned to a ^{3}IL ($\pi \rightarrow \pi^*$) (ppy-COOH) state. Interestingly, this complex displayed biexponential decay (τ_o = 1.78 and 0.48 μs) in MeOH. We have tentatively assigned the newly emerged shorter-lived component to a ^{3}MLCT/^{3}LLCT state, which should result from the interaction between ppy-COOH and MeOH molecules.[7a] Additionally, the presence of an electron-donating oxymethyl unit on the bpy ligand substantially increased the emission lifetimes and quantum yields (Table 1). The second-order rate constants for the reaction of complex **3** with benzyl azide were determined to be $6.9 \pm 0.9 \times 10^{-2}$ M^{-1} s^{-1} in CD_3OD and $5.1 \pm 0.1 \times 10^{-2}$ M^{-1} s^{-1} in $(CD_3)_2CO$ (ESI‡), which are comparable to those of complex **2** and other DIBO derivatives.[3b] Also, complex **3** underwent facile conjugation with azide-modified BSA, HSA, and aTf, affording conjugates that showed intense and long-lived phosphorescence upon excitation (ESI‡).

Due to the polar ppy-COOH ligand and TEG linker, the lipophilicity of complex **3** (log $P_{o/w}$ = 5.20) was lower than that of complexes **1** and **2** (ESI‡). Additionally, MTT assays showed that complex **3** was noticeably less cytotoxic than complexes **1** and **2** (ESI‡). All these properties indicate that complex **3** has high biocompatibility and is a very promising candidate for *in vivo* applications. Ac_4ManNAz-treated and -untreated CHO cells were incubated with complex **3** prior to analysis by confocal microscopy, flow cytometry, and ICP-MS. Interestingly, we found that Ac_4ManNAz-treated cells exhibited intense emission from the cell membrane plus some internal compartments (Fig. 2). In sharp contrast, the untreated cells did not show any emission, highlighting the bioorthogonal labeling characteristics of complex **3**. Flow cytometry revealed that the pretreatment enhanced the emission intensity of CHO cells by *ca.* 8.0 fold and ICP-MS measurements showed that the uptake was increased by *ca.* 3.6 times (ESI‡). We believe that the observed emission of the internal compartments is due to the intracellular trafficking of the labeled glycans (Fig. 2); similar observations have been reported for other fluorescent bioorthogonal probes.[3a] The emission intensity of CHO cells treated with complex **3** (100 μM, 37 °C, 1 h) was maintained at 99.7% upon constant irradiation at 405 nm by a confocal laser (25 mW) for 2 min, indicative of the high photostability of the probe.

In conclusion, three cyclometalated iridium(III) polypyridine complexes appended with a DIBO pendant have been designed as the very first phosphorescent bioorthogonal labeling reagents for azide-modified biomolecules. Although many transition metal complexes exhibit inherent cytotoxicity,[5,6,7b] we have shown that this can be minimized by using more polar ligands and substituents such as PEG,[7c] with the intriguing emission behavior being preserved. Related work on phosphorescent bioorthogonal probes for imaging and therapeutic applications is in progress.

We thank The Hong Kong Research Grants Council (Project No. CityU 102212 and CityU 102410) for financial support. S.P.-Y.L. acknowledges the receipt of a Postgraduate Studentship, a Research Tuition Scholarship, and an Outstanding Academic Performance Award, and T.S.-M.T acknowledges the receipt of a Postgraduate Studentship, all administered by City University of Hong Kong.

Notes and references

1 (*a*) E. Saxon and C. R. Bertozzi, *Science*, 2000, **287**, 2007; (*b*) J. A. Prescher and C. R. Bertozzi, *Nat. Chem. Biol.*, 2005, **1**, 13; (*c*) P. V. Chang, J. A. Prescher, M. J. Hangauer and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2007, **129**, 8400; (*d*) E. M. Sletten and C. R. Bertozzi, *Angew. Chem., Int. Ed.*, 2009, **48**, 6974.

2 V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596; C. W. Tornøe, C. Christensen and M. Meldal, *J. Org. Chem.*, 2002, **67**, 3057.

3 (*a*) J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 16793; (*b*) X. Ning, J. Guo, M. A. Wolfert and G.-J. Boons, *Angew. Chem., Int. Ed.*, 2008, **47**, 2253; (*c*) J. Dommerholt, S. Schmidt, R. Temming, L. J. A. Hendriks, F. P. J. T. Rutjes, J. C. M. van Hest, D. J. Lefeber, P. Friedl and F. L. van Delft, *Angew. Chem., Int. Ed.*, 2010, **49**, 9422; (*d*) J. C. Jewett, E. M. Sletten and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2010, **132**, 3688.

4 See, for example, A. Bernardin, A. Cazet, L. Guyon, P. Delannoy, F. Vinet, D. Bonnaffé and I. Texier, *Bioconjugate Chem.*, 2010, **21**, 583.

5 R. Blasius, C. Moucheron and A. Kirsch-De Mesmaeker, *Eur. J. Inorg. Chem.*, 2004, 3971; V. Fernández-Moreira, F. L. Thorp-Greenwood and M. P. Coogan, *Chem. Commun.*, 2010, **46**, 186; Q. Zhao, C. Huang and F. Li, *Chem. Soc. Rev.*, 2011, **40**, 2508; K. K.-W. Lo, A. W.-T. Choi and W. H.-T. Law, *Dalton Trans.*, 2012, **42**, 6021; M. Patra and G. Gasser, *ChemBioChem*, 2012, **13**, 1232; E. Baggaley, J. A. Weinstein and J. A. G. Williams, *Coord. Chem. Rev.*, 2012, **256**, 1762.

6 K. K.-W. Lo, M.-W. Louie and K. Y. Zhang, *Coord. Chem. Rev.*, 2010, **254**, 2603; K. K.-W. Lo, K. Y. Zhang and S. P.-Y. Li, *Eur. J. Inorg. Chem.*, 2011, 3551; K. K.-W. Lo and K. Y. Zhang, *RSC Adv.*, DOI: 10.1039/C2RA20967E.

7 (*a*) K. K.-W. Lo, K. Y. Zhang, S.-K. Leung and M.-C. Tang, *Angew. Chem., Int. Ed.*, 2008, **47**, 2213; (*b*) K. Y. Zhang, S. P.-Y. Li, N. Zhu, I. W.-S. Or, M. S.-H. Cheung, Y.-W. Lam and K. K.-W. Lo, *Inorg. Chem.*, 2010, **49**, 2530; (*c*) S. P.-Y. Li, H.-W. Liu, K. Y. Zhang and K. K.-W. Lo, *Chem.–Eur. J.*, 2010, **16**, 8329.

8 A. J. Amoroso, R. J. Arthur, M. P. Coogan, J. B. Court, V. Fernádez-Moreira, A. J. Hayes, D. Lloyd, C. Millet and S. J. A. Pope, *New J. Chem.*, 2008, **32**, 1097; M.-W. Louie, H.-W. Liu, M. H.-C. Lam, Y.-W. Lam and K. K.-W. Lo, *Chem.–Eur. J.*, 2011, **17**, 8304.