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Luminescent oligo(ethylene glycol)-functionalized cyclometalated platinum(II) complexes: cellular characterization and mitochondria-specific localization†

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A readily tunable series of non-planar oligo(ethylene glycol)-substituted phosphorescent Pt(II) complexes has been investigated as live cell imaging agents; suitable structural modifications can give good cellular uptake, traceable mitochondria-specific localization and potent cytotoxic characteristics towards HeLa cells.

The design of molecular optical imaging probes that exhibit traceable cellular localization is a challenging endeavor. Transition metal–organic[1] (and lanthanide[2]) complexes offer great promise as diagnostic and therapeutic agents due to their modularity and adaptability, well-defined structure and coordination geometry, availability of potential binding sites, and the possibility of visible-light and phosphorescent signaling from tunable, diverse excited states (without interference from organic/background fluorescence).[3] However, although a rational relationship between molecular structure and cellular uptake/localization may be anticipated and would be highly beneficial for application studies, this level of understanding remains unrealized.

Previous studies concerning luminescent platinum(II) bioprobes have largely employed the "planar lipophilic cation" design strategy. Two related Pt(II) systems, supported by cyclometalated [6-aryl-2,2′-bipyridine and mono-(N-heterocyclic carbene)][4] or [2-arylpyridine and bis-(NHC)][5] ligand sets, were reported to display contrasting cellular localization, namely to mitochondria and the endoplasmic reticulum (ER) respectively, although both classes show cytotoxic behavior. The application of a PPh_3-containing cationic cyclometalated Pt(II) luminophore as a bioimaging agent was investigated;[6] this complex was expected to target the mitochondria (see below), but nucleolus-specific localization was observed. Similar preferential staining of the nucleolus was detected for the neutral tridentate cyclometalated [2,6-di(2′-pyridyl)phenyl]PtCl complex, which displays low cytotoxicity.[7] On the other hand, reports on anionic Pt(II) probes have revealed distribution within the cytoplasm (with partial localization in the perinuclear region)[8] or staining of the plasma membrane.[9]

Mitochondria are the primary cellular producers of ATP and play important roles in the cell cycle, regulation of metabolism, and signaling and programmed cell death (apoptosis).[10] Hence, mitochondria are attractive targets for the development of drug leads with superior characteristics and reduced side effects, and the integration of a luminescence reporting element would facilitate visualization and controllable targeting. Organic fluorophores that display selective mitochondrial staining are well-established and typically rely upon cationic triphenylphosphonium or ammonium functionalities.[11] Recently, reports on mitochondria-specific phosphorescent and metal–organic probes [Re(I),[12] Ru(II),[13] Ir(III),[14] Pt(II);[4] Eu(III)[15]] have begun to emerge.

We are currently engaged in the development of functionalized phosphorescent complexes featuring environmentally responsive Pt(II) moieties, with the aim of creating molecular assemblies with unusual photophysical and sensing characteristics that allow the reporting of molecular-level perturbations and events.[16,17] Herein, we describe the synthesis and spectroscopic properties of a readily tunable family of oligo(ethylene glycol)-substituted cyclometalated Pt(II) complexes, and investigate their applications as live cell imaging agents. We demonstrate that suitable structural modifications can confer mitochondria-specific localization and potent cytotoxic characteristics to these non-planar cationic probes.

A versatile synthetic route was devised for the Pt(II) complexes (Scheme 1; see the ESI† for details); the oligo(ethylene glycol) chain was incorporated to improve biocompatibility, while the 4-phenyl moiety at the central pyridine ring was designed to augment emission efficiency.[18] For example, a modified Kröhnke-type reaction was performed to generate 4-phenyl-6-phenol-2,2′-bipyridine, and substitution yielded the key 4-phenyl-6-aryl-2,2′-bipyridine precursor bearing a tosylate-terminated tetra(ethylene glycol) substituent, which readily undergoes substitution with phenols and secondary amines to give the desired series of ligands. Subsequent metalation with K_2PtCl_4 in refluxing glacial acetic acid, followed by treatment with PPh_3 in CH_3CN/CH_3OH (1 : 1) and salt metathesis using $LiClO_4$, afforded the cationic complexes **3–7** with different

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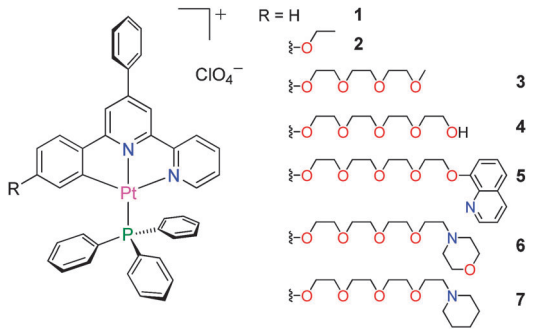

Scheme 1 Structures of platinum(II) complexes.

oligo(ethylene glycol)-appended functionalities and lipophilicities (see below). The parent (**1**) and ethoxy (**2**) derivatives have been prepared for comparative studies. All complexes have been characterized by NMR spectroscopy, mass spectrometry and elemental analysis. For example, the $^{31}P\{^{1}H\}$ NMR spectrum of **7** displays a singlet signal at 25.5 ppm, flanked by characteristic ^{195}Pt satellites with a $^{1}J_{P,Pt}$ value of 4000 Hz, which matches the corresponding coupling for the doublet peak in the ^{195}Pt NMR spectrum at −4143 ppm (Fig. S1, ESI†).

The photophysical properties of complexes **1–7** have been investigated by UV-vis absorption and emission spectroscopy (Fig. 1 and Table S1, ESI†). The visible absorption bands of **2–7** at wavelengths >400 nm are tentatively assigned to an admixture of π(alkyl-*O*-aryl)/d(Pt) → π*(bpy) (^{1}ILCT/^{1}MLCT), following previous studies on neutral alkoxy congeners[17c,19] and in contrast to the d(Pt) → π*(bpy) (^{1}MLCT) transition of **1**.[18a] All complexes in this study are luminescent in solution at room temperature. Compared with the ^{3}MLCT emission of **1** (λ_{max} 534 nm in CH_3CN), the emission bands of **2–7** are red-shifted to *ca.* λ_{max} 580 nm and exhibit minor solvatochromism (for **3**: Fig. S2, ESI†), which is indicative of an ^{3}ILCT excited state, although mixing with ^{3}MLCT is likely.[17c,19] As a representative example, the structure of **3** was optimized by DFT calculations (Fig. S3, ESI†). The electron densities of the HOMO and LUMO from the energy-minimized calculated (Gaussian) structure are shown to be primarily localized on the 6-(alkyl-*O*)-aryl-2,2′-bpy ligand and to a small extent on the Pt atom (and not on the 4-phenyl or PPh_3 moieties), thus consistent with the above assignment.

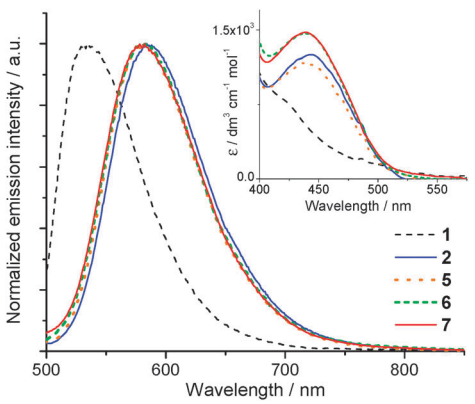

Fig. 1 Normalized emission spectra (inset: low-energy UV-vis absorption bands) of selected complexes (10^{-5} M; spectra of **3** and **4** closely matches **5–7** and are omitted for clarity) in CH_3CN at 298 K.

The intracellular distribution of the Pt(II) probes has been investigated using laser-scanning confocal microscopy. The microscopy images of HeLa cells incubated with each complex (2.5 μM) at 37 °C for 1 hour were obtained. Intracellular emissions were observed upon excitation at 405 nm, confirming that these cationic oligo(ethylene glycol)-substituted complexes can be internalized by live cells. Complexes **1–5** were distributed in the cytoplasm with some degree of punctate staining in the perinuclear region (Fig. S4, ESI†), but moderately weak emissions were detected in each case (including for the quinoline derivative **5**), which hampered further subcellular characterization. Upon changing to the morpholine (**6**) and piperidine (**7**; Fig. 2a) analogues, strong emissions with intense punctate staining of HeLa cells were detected. The emission spectra of **6** and **7** obtained from HeLa cells display good resemblance to those in solution (Fig. S5, ESI†), strongly suggesting that the Pt(II) luminophores remain intact inside the cells. The weaker emissions of **1–5** should not be ascribed to poor cellular uptake, since the intracellular amounts of platinum for all complexes fall within a comparable level of 10^{-16} mole (see Table 1).

To evaluate possible organelle-specific localization, the confocal microscopy images of HeLa cells co-incubated with **7** (and **6**) and various commercially available fluorescent dyes, namely MitoTracker Green FM (for mitochondria; Fig. 2b for **7**), LysoTracker Green DND-26 (for lysosomes), ER Tracker Green (for ER domains) and Hoechst 33342 (for DNA), were examined. The confocal images of **7** and MitoTracker Green FM are closely superimposable (Fig. 2c), indicating that these two dyes are co-localized, which is also the case for **6** (Fig. S6, ESI†). No significant co-localization was found for **6** or **7** (Fig. S7 and S9, respectively, ESI†) with the lysosome-, ER- and nucleus-specific dyes. The lack of significant co-localization for **6** and LysoTracker is of interest, bearing in mind the established ability of alkylmorpholine to target the acidic nature of lysosomes.[20] The complete absence of co-localization for **6** or **7** and the DNA binder Hoechst 33342 is also intriguing, considering the known propensity of square planar Pt(II) complexes to accumulate in the nucleus by cross-linking and/or intercalation,[6,7,21] and we attribute this to their non-planar structures imposed by the 4-phenyl and bulky PPh_3 moieties.

The intensity profiles of the linear regions of interest across HeLa cells stained with **6** (and **7**) and MitoTracker Green FM (Fig. S6 and S8, respectively, ESI†) display good overlap. The co-localization of **6** and **7** with the fluorescent dyes was quantified by correlation analysis. The Pearson coefficients (*Rr*) of co-localization for **6** with MitoTracker, LysoTracker and ER Tracker are 0.80, 0.64 and 0.55, respectively, while those for **7** are 0.73, 0.58 and 0.38, respectively. These results indicate that complexes **6** and **7** can preferentially localize in the mitochondria of HeLa cells. Incubation of HeLa cells with **6** and **7** at 4 °C resulted in a very weak emission (Fig. S10, ESI†), indicating that the complexes entered the cells through an energy-dependent endocytosis pathway. In a time-dependent MTT assay, minimal decreases of cell survival percentage were recorded after incubation of HeLa cells with **6** and **7** at 37 °C for 8 hours at a dose concentration of 2.5 μM (Fig. S11, ESI†). Control experiments have been undertaken to investigate the intracellular localization of the corresponding neutral derivatives of **3**, **6** and **7** (where PPh_3 is replaced by Cl), but no emission was detected after incubation with HeLa cells (presumably due to minimal uptake).

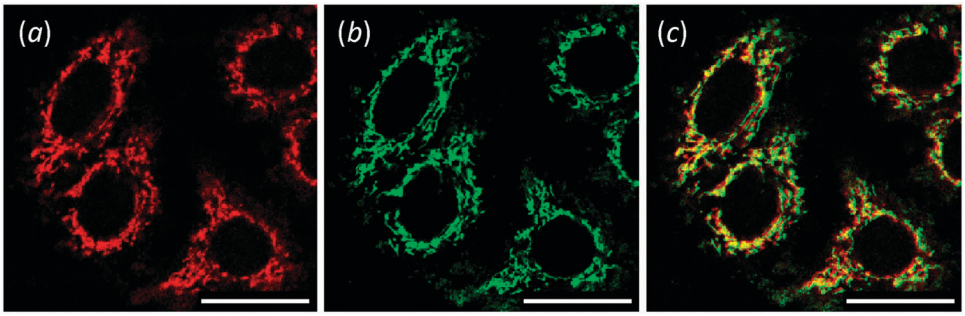

Fig. 2 Confocal microscopy images of HeLa cells incubated with (a) **7** (2.5 μM, 1 hour at 37 °C; λ_{ex} = 405 nm, λ_{em} = 580–750 nm) and (b) MitoTracker Green FM (25 nM, 20 min; λ_{ex} = 488 nm, λ_{em} = 520–560 nm); (c) merged images of (a) and (b). Scale bar = 25.0 μm.

Table 1 Lipophilicity ($\log P_{o/w}$), cellular uptake and cytotoxicity (IC_{50}) of **2–7** towards HeLa cell lines

Complex	$\log P_{o/w}$[a]	Intracellular amt[b]/fmol	IC_{50}[c]/μM
2	6.1 ± 0.13	0.63 ± 0.06	2.4 ± 0.06
3	5.2 ± 0.12	0.27 ± 0.09	9.4 ± 1.6
4	5.4 ± 0.12	0.58 ± 0.09	10.0 ± 1.7
5	5.9 ± 0.12	0.71 ± 0.13	9.2 ± 1.0
6	5.5 ± 0.12	0.75 ± 0.09	8.8 ± 0.6
7	4.0 ± 0.02	0.83 ± 0.07	10.0 ± 0.5
Cisplatin	−2.30[d]	n.d.[e]	100 ± 4.6

[a] See ref. 22. [b] Amount of platinum associated with average HeLa cells upon incubation with complex (5.0 μM) at 37 °C for 1.5 h; determined by ICP-AES. [c] For HeLa cells incubated with complex at 37 °C for 24 h. [d] From ref. 23. [e] Not determined.

Data on lipophilicity, cellular uptake and cytotoxicity have been collated (Table 1). The lipophilicity of a biological probe is a critical attribute that strongly influences cellular uptake, intracellular localization and cytotoxicity,[13,24] and it has been demonstrated that lipophilic cations accumulate in mitochondria as a result of the negative potential difference across the mitochondrial membrane.[25] The lipophilicity ($\log P_{o/w}$) of the complexes has been evaluated by reversed-phase HPLC and values of 4.0 (for **7**) to 6.1 (for **2**) have been determined.

In spite of the comparable cellular uptake of **2–7**, and similar lipophilicity values of **3–5** and **6**, the intracellular emissions of **6** and **7** are evidently more intense. It should be noted that emission quenching by endogenous species such as glutathione (GSH), present at near-millimolar concentrations, would be prevalent under normal circumstances.[7,26] We tentatively postulate that the "unquenched" emissions of **6** and **7** may be considered to arise from enhancement or restoration of luminescence upon preferential binding and localization at hydrophobic domains[27] in mitochondria. In support of this, preliminary studies have been performed in which emission quenching of **6** and **7** by GSH in PBS buffer, and the subsequent ability of the model protein bovine serum albumin (BSA; and lipophilic domains therein) to significantly restore the emission intensity, have been demonstrated (Fig. S12, ESI†). The clearly observable mitochondria-specific localization may be ascribed to various factors including suitability of lipophilicity and functional group (possibly basic[28]). In general, the complexes display potent cytotoxicity (IC_{50}) against HeLa cells, which is higher than that for cisplatin by at least a factor of 10. Many mitochondria-specific phosphorescent dyes are reported to exhibit pronounced cytotoxic activity, and it is feasible that the observed toxicity (especially for **6** and **7**) also stems from mitochondrial accumulation[29] causing dysfunction or damage leading to cell death, although alternative pathways should be considered.

In summary, suitable derivatization and variation in lipophilicity of luminescent non-planar oligo(ethylene glycol)-functionalized platinum(II) complexes have been shown to give traceable mitochondria-specific localization in HeLa cells, and preliminary results have been obtained which provide support for the interpretation regarding imaging studies (*i.e.* emission quenching upon uptake; restoration in hydrophobic domains). Future work will aim to exploit the readily derivatizable nature of this system to gain greater insight into the factors affecting cellular uptake and localization, as well as investigate the origin of the cytotoxicity.

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