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Two-Photon Plasma Membrane Imaging in Live Cells by an Amphiphilic, Water-Soluble Cyctometalated Platinum(II) Complex

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An amphiphilic, water-soluble cyclometalated Pt(II) complex with two-photon emission properties has been developed as a molecular marker specific for in vitro plasma membrane staining.

In comparison to single-photon bioimaging techniques, multiphoton imaging with near-infrared excitation offers a number of advantageous features including the reduction of phototoxicity to biological samples, the lowering of scattering, and the inherent capability of optical sectioning.[1,2] The increasing popularity of multiphoton microscopy has prompted the development of new fluorescent probes to meet the needs of a variety of bioimaging objectives. Up to now, a majority of the commercially available two-photon dyes have been organic compounds with broad emission spectra and short fluorescent lifetimes that are difficult to distinguish from biological autofluorescence.[3] In this context, research on coordination/organometallic complexes as biocompatible multiphoton luminescent probes and markers has emerged in recent years, and these have already found numerous biological applications.[4−8] Cyclometalated platinum(II) complexes constitute one of these promising organometallic-based multiphoton bioimaging systems.[9,10]

Lipophilic cyanine and styryl dyes are commonly used in the staining of cell membranes. However, they are sometimes difficult to apply and can be rapidly internalized. Only a few of these markers, for example, 1,1′-dioctadecy-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DilC18(7)) and *N*-(3-triethylammoniumpropyl)-4-(4(dibutylamino)styryl)pyridinium dibromide (FM 1−43), are able to be excited in the near-infrared range.[11] We have recently reported the two-photon-induced luminescent properties of a cyclometalated Pt(II) complex [Pt($\mathbf{L_1}$)Cl] (**1**), with a special $C_{phenyl}N_{pyridyl}N_{pyrazolyl}$ cyclometalating ligand H$\mathbf{L_1}$ (2-phenyl-6-(1*H*-pyrazol-3-yl)-pyridine), and demonstrated its potential in two-photon live cell imaging.[10] By simple modification of its ligands, this cycloplatinated complex system can be fine-tuned into multiphoton bioimaging probes with different organelle specificities. In this work, we report a rational modification of the $C_{phenyl}N_{pyridyl}N_{pyrazolyl}$ and ancillary ligands of **1** to convert it into a plasma-membrane-specific two-photon marker. The derivatization of H$\mathbf{L_1}$ to incorporate a hydrophobic C18 chain and the replacement of the ancillary chloride ligand on **1** by a water-soluble *p*-trisulfonated triphenylphosphine ($[PPh_3\text{-}3SO_3]^{3-}$) have led to an anionic, water-soluble complex, [Pt($\mathbf{L_2}$)(PPh_3-$3SO_3$)]$^{2-}$ (**2**) (as sodium salt), with amphiphilic characteristics resembling anionic surfactants (e.g., SDS; Scheme 1). This new cycloplatinated complex, **2**, is able to stain and remain localized in the plasma membrane of live cells over extended periods of time. To the best of our knowledge, **2** is the first example of a water-soluble organometallic complex which is able to

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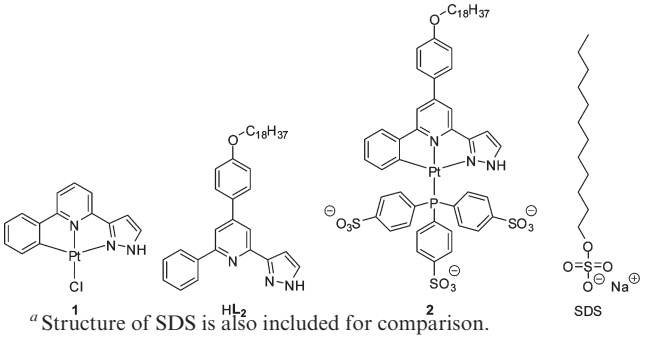
Scheme 1. Molecular Structures of the Cycloplatinated Two-Photon *In Vitro* Bioimaging Dyes **1** and **2** as well as the Modified Hydrophobic Cyclometalating Ligand HL_2[a]



[a] Structure of SDS is also included for comparison.

specifically localize in the plasma membrane for two-photon imaging purposes.

The electronic transition spectrum of **2** in a pH 7.0 HEPES buffer at 298 K is depicted in Figure 1a. The strong absorption features at ca. 300−400 nm with extinction coefficients (ε) on the order of 10^4 dm^3 mol^{-1} cm^{-1} were tentatively assigned the spin-allowed singlet intraligand ^{1}IL (π(L) → π*(L)) transitions, which have probably been overlapped by the n(L) → π*(L) transitions involving the oxygen of the alkoxyl fragment.[12] Absorption in the 400−450 nm region was small (ε < 1000 dm^3 mol^{-1} cm^{-1}). As suggested by Che and co-workers in their studies on the analogous [Pt($C^{\wedge}N^{\wedge}N$)PPh$_3$]$^+$ ($HC^{\wedge}N^{\wedge}N$ = 6-phenyl-2,2′-bipyridine, PPh$_3$ = triphenylphosphine),[13] the higher electronic charge of the PPh$_3$ substituted complexes pushed the metal-to-ligand charge-transfer (MLCT) transition to higher energies, causing the overlapping of the ^{1}MLCT absorption bands with the ^{1}IL absorption bands.

The cycloplatinated complex **2** produced strong luminescence in neat aqueous media (HEPES buffer at pH 7.0) at room temperature. Upon linear excitation at 355 nm, **2** displayed a poorly resolved emission band with λ_{max} at 528 nm (Figure 1b). The large Stoke's shift and relatively long emission lifetime (1.95 μs) suggested that the emission was originated from a spin-forbidden triplet excited state. With reference to [Pt($C^{\wedge}N^{\wedge}N$)Cl], this emission band was tentatively assigned the ^{3}MLCT emission, although some degree of mixing with the ^{3}IL character could not be ruled out. The luminescence quantum yield, ϕ_{lum}, of **2** in the aqueous media was measured to be 0.30.

Complex **2** only possessed linear absorption in the UV region but not at wavelengths longer than 500 nm. Upon femto-second excitation at λ_{ex} = 720 nm, bright green emission (λ_{max} = 528 nm) was observed. The band shape and location of this emission strongly resembled its single-photon luminescence. This suggests that these emissions are of a similar origin. Furthermore, power dependence experiments revealed a log−log linear relationship between the emission peak intensity and the incident excitation power at 720 nm with a slope of 2.01 (Figure 1d). This confirms the two-photon excitation process. With reference to Rhodamine 6G,[14] the two-photon cross-section of **2** was estimated to be ca. 88 GM in aqueous media (HEPES buffer of pH 7.0). The two-photon cross-section of **2** is highly comparable to those commercially available two-photon membrane stains. For example, 5-hexadecanoylaminofluorescein, a lypophilic two-photon membrane dye, contains a fluoroscein fluorophore that possesses a two-photon cross-section, measured in water, of 38 GM upon excitation at 780 nm.[15] Kim et al. reported a series of lypophilic two-photon cell membrane probes based on 6-acyl-2-aminonaphthalene with two-photon absorption cross-sections in the range 85−150 GM at 780 nm in ethanol.[16]

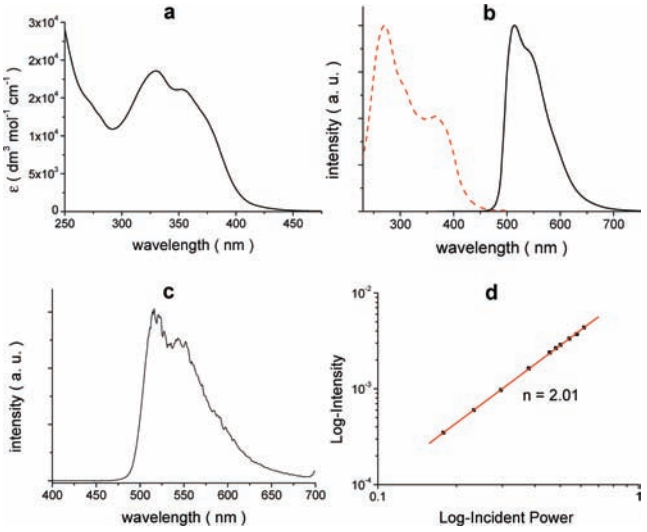


Figure 1. (a) Linear UV−vis absorption spectrum of **2** ($5 \times 10-5$ M) in HEPES buffer at pH of 7.0. (b) Linear excitation (red dotted line) and emission (black line) spectra of **2** (5×10^{-5} M) in HEPES buffer at pH of 7.0. (c) Two-photon emission spectrum of **2** (1×10^{-4} M) in HEPES buffer at pH of 7.0; λ_{ex} = 720 nm from a femto-second mode-locked Ti:sapphire laser. (d) Power dependence measurements showing n = 2.01.

Resembling its parent complex [Pt($\mathbf{L_1}$)Cl] (**1**), complex **2** was rapidly taken up and accumulated in the cells. All images were readily observable without prior washing to remove the excess dye in the medium. This is very different from many of the commercially available fluorescent dyes, which function solely by diffusion. A bioaccumulative dye requires a much lower loading of dye and a much shorter incubation time of cell staining. All of these are favorable properties for time-lapse imaging, which aims at monitoring the cell morphology under different experimental conditions.

Linear and two-photon in vitro live cell imaging were carried out on the human cervical carcinoma (HeLa) cell line at a low dosage of **2** (1 μg/mL, Figure 2). With the long hydrophobic C18 carbon chain, **2** showed rapid and efficient localization in the plasma membrane of live cells, giving

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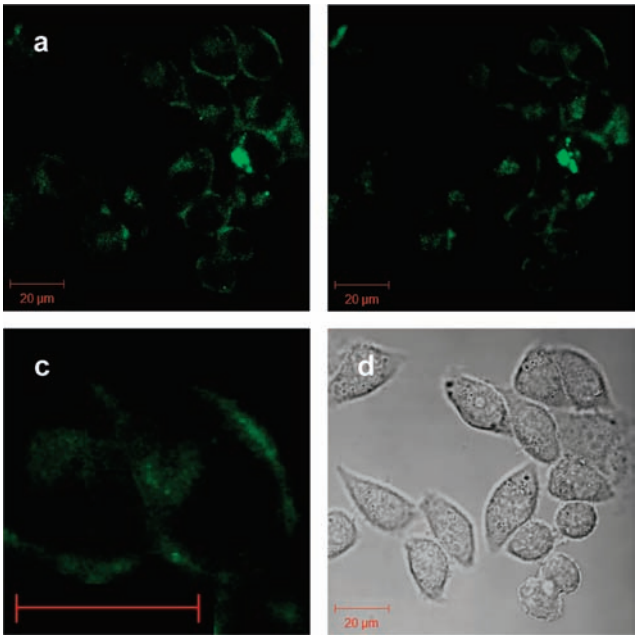

Figure 2. (a) Linear confocal microscopy image ($\lambda_{ex} = 400$ nm) of Hela cells after 30 min of exposure to **2** (1 μg/mL in the culture medium). (b) Two-photon ($\lambda_{ex} = 720$ nm, filter band-pass for $\lambda_{em} = 500-550$ nm) confocal microscopy image after 30 min of exposure to **2**. (c) A 3.5× magnified duplicate of b. (d) Bright field image of a and b. (scale bar = 20 μm).

strong green emission under linear and two-photon excitations at 400 nm (Figure 2a) and 720 nm (Figure 2b), respectively, to reveal clear plasma membrane images of the Hela cells. The quality of membrane images under linear excitation and two-photon excitation are similar. The magnified image in Figure 2c clearly showed the localization of **2** in the plasma membrane.

Besides enhancing the water solubility of **2**, the *p*-trisulfonated triphenylphosphine ancillary ligand is also believed to help maintain the localization of the complex in the plasma membrane. Time lapse experiments (see the Supporting Information) indicated that **2** can remain in the plasma membrane for at least 3 h with no subcellular migration to the cyctoplasm.

Complex **2** showed minimal cytotoxicity to both HeLa and normal epithelial cells at a concentration 10-fold higher than the normal dosage for imaging (i.e., 10 μg/mL) (refer to the Supporting Information). For HeLa cells, ca. 90% cell viability was observed after 25 h of exposure. Even for the more vulnerable normal epithelial cells, ca. 70% cell viability was observed after 25 h of exposure.

To conclude our findings, we have demonstrated the versatility of the $C_{phenyl}N_{pyridyl}N_{pyrazolyl}$ cyclometalated platinum(II) system in multiphoton bioimaging. Desirable biolabeling properties can be achieved by rational modification of the ligands. With the incorporation of both a hydrophobic long carbon chain onto the cyclometalating ligand and the coordination of a multiple anionic, water-soluble ancillary ligand, an amphiphilic, water-soluble cycloplatinated two-photon marker with remarkable plasma membrane staining properties was obtained. The fast uptaking of the complex by live cells, the long resident lifetime in the plasma membrane, the high two-photon induced luminescent intensity, and the low cytotoxicity make complex **2** a potentially useful tool in plasma-membrane-related studies.

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Supporting Information Available: Detailed synthetic procedures and characterization data for **2**; cytotoxicity assays of **2** in HeLa and normal epithelial cells and time-lapse linear in vitro microscopy studies with **2** in HeLa cells. This material is available free of charge via the Internet at http://pubs.acs.org.