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Excitation energy transfer in ruthenium (II)-porphyrin conjugates led to enhanced emission quantum yield and $^1O_2$ generation

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ABSTRACT

Porphyrins are good photodynamic therapy (PDT) agents due to its flexibility for modifications to achieve tumor localization and photo-cytotoxicity against cancer. Yet they are not perfect. In a Ru(polypyridyl)-porphyrin system, the Ru(polypyridyl) moiety improves the water solubility and cell permeability. Consider the similar excited state energies between Ru(polypyridyl) and porphyrin moieties; a small perturbation (*e.g.* Zn(II) metalation) would lead to a marked change in the energy migration process. In this work, we have synthesized a series of porphyrins conjugated with Ru(polypyridyl) complexes using different linkers and investigated their photophysical properties, which included singlet oxygen quantum yield and their in vitro biological properties, resulting from linker variation and porphyrin modification by Zn(II) metalation.

1. Introduction

Porphyrins and its analogues have been widely used as photosensitizers in anti-cancer photodynamic therapy (PDT) owing to their preferential accumulation in tumour cells, *i.e.*, *ca.* 2- to 5-fold higher than that in normal cells and their ability to induce oxidative damage to various vital cellular components, such as DNA, proteins and membrane components, thus causing cell death [1–5]. However, their applications are hindered by poor water-solubility and low cell-permeability. It was found out that molecule which is amphiphilic and cationic presenting better cell permeability [6,7]. In the literature, Ru(II)-polypyridine complexes have been shown to cause potent damage to tumour cells through their intercalation into double-stranded DNA and RNA, thus interfering with the strand separation during cell division [8–10]. In fact, some ruthenium complexes have recently been developed as anti-cancer PDT agents as well [11,12]. Recently, Tsuge and coworkers have found that energy transfer in porphyrin-Ru conjugates takes place from the Ru(II) ^{3}MLCT state to the porphyrin singlet state; but an introduction of Zn(II) ion into the porphyrin can increase its excited-state energy level, resulting in a reversal of the direction of energy transfer [13,14]. In fact, controlled manipulation of the energy flow in photo-/redox-active supramolecular systems is crucial to diverse research fields, ranging from artificial photosynthesis, photocatalysis, photovoltaics as well as PDT [15–17]. Many parameters, such as light, redox potential, solvent used, pH, the effects of co-existing ligand and nearby ions have been found to play a role [18,19]. In our earlier study, we examined the effect of linker on the properties of porphyrin-ruthenium conjugates [20]. The conjugate with a rigid linker has been shown to have the most promising *in vitro* photobiological properties for further development as a bifunctional probe for two-photon (NIR) induced PDT and tumour imaging. However, the excitation energy transfer mechanism between these components needs to be elucidated, particularly when the excited-state energies of the porphyrin and the Ru-polypyridine moieties are very close. Our objective is to design novel three-component (*i.e.*, porphyrin-linker-Ru(II) complex) supra-molecular systems for tumour imaging and PDT applications. Different substituent groups on the porphyrin and the choice of linkers can potentially exert significant effects on the water solubility, cellular uptake, subcellular localization, and more importantly, the direction and efficiency of excitation energy transfer in these conjugates. A comprehensive evaluation of the luminescence quantum yields and PDT efficacies will furnish important data for an understanding of the energy transfer mechanism and for achieving an optimised design for PDT and tumour imaging applications of these conjugates (Fig. 1).

In this work, two water-soluble free-base porphyrin-Ru(II) conjugates, **1–2**, and two Zn(II) porphyrin-Ru(II) conjugates, **3–4**,

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with flexible non-π-conjugative and rigid π-conjugative linkers incorporated between the hydrophobic porphyrin moiety and the hydrophilic Ru(II)-polypyridyl complex have been synthesized. The energy transfer mechanism in these porphyrin-ruthenium conjugates has been discussed in detail. As previous expected, the introduction of Zn(II) into the as-prepared free-base porphyrin-Ru (II) conjugates results in re-direct the excitation energy flow and it can be fully confirmed by a comprehensive studies of their UV–vis absorption and emission spectra. Their $^1O_2$ and emission quantum yields were measured and compared, which show that the free-base porphyrin-Ru(II) conjugates, **1–2**, affords a higher $^1O_2$ quantum yield, relative to the two Zn(II) porphyrin-Ru(II) conjugates, **3–4**. In *in vitro* studies, **1** and **2** shown stronger PDT activities than **3–4**, presumably due to their higher singlet oxygen quantum yields.

2. Experimental sections

2.1. General information for syntheses

The synthetic routes for the preparation of the porphyrin-Ru (pyridyl) conjugates are shown in Scheme S1. Asymmmetric porphyrins were synthesized by adding pyrrole and substituted benzaldehyde in propionic acid and refluxed for 3 h. Reaction of the porphyrin with bromoacetyl bormide in the presence of catalytic amount of Cs_2CO_3 in anhydrous DMF gave **L1** in ~50% yield. **L2** was synthesized with high yield (~90%) by Schiff-based formation. Condensation of the corresponding diketone phenanthroline-derivatives with aldehydes in the presence of ammonium acetate in acetic acid, afforded imidazole-linked porphyrin (**L2**). The complexation reaction of *cis*-$Ru(bpy)_2Cl_2$ with **L1** and **L2** followed by reaction with $Zn(OAc)_2$ gave conjugates **1–4** respectively in good yield (~85%). The detailed information of synthesis for all intermediates and products were described in the Supplementary material.

2.2. Photophysical measurement

UV-visible absorption spectra (200–1100 nm) were recorded by an HP Agilent UV-8453 spectrophotometer. Single-photon luminescence spectra were recorded using an Edinburgh Instrument FLS920 combined fluorescence lifetime and steady state spectrophotometer that was equipped with a visible to near-infrared sensitive photomultiplier in nitrogen-cooled housing. The spectra were corrected for detector response and stray background light phosphorescence. The quantum yields of the compounds were measured by the comparative method and integrated sphere [18]. Singlet oxygen was detected directly by its phosphorescence emission at 1270 nm using an InGaAs detector on a PTI QM4 luminescence spectrometer. The emission quantum yields (Φ_{em}) of the test compounds were measured by comparative method and integrated sphere according to Eq. (1) (H_2TPP, $\Phi_{em}=0.11$ and ZnTPP, $\Phi_{em}=0.033$ in toluene, Abs=0.05) [20]. Singlet oxygen quantum yield (Φ_Δ) were determined in chloroform by comparing their singlet oxygen phosphorescence emission signals to that of a reference compound (H_2TPP, $\Phi_\Delta=0.55$ in chloroform) [21,22].

$$\Phi_{em}^{S}=\Phi_{em}^{REF}\times\left(\frac{n_S}{n_{REF}}\right)^2\frac{G_\Delta^S}{G_\Delta^{REF}}\times\frac{A_{REF}}{A_S} \quad (1)$$

where Φ_{em} stands for emission quantum yield, G_Δ represents the integrated emission intensity, A indicates the absorbance at the excitation wavelength, and n is the refractive index of the solvent. REF and S correspond to the reference and the sample, respectively.

2.3. Cell culture

Human cervical carcinoma HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C and 5% CO_2.

2.4. Flow cytometric cellular uptake

HeLa cells were seeded onto wells of a 6-well plate overnight. The cells were then incubated with 10 μM complexes **1–4** for 6 or 24 h at 37 °C and 5% CO_2. Cells were then trypsinized and washed with 1X PBS for twice. The uptakes were then monitored with flow cytometer (BD FACSaria Cell Sorting System, BD Biosciences, China). The cells were excited by a 633 nm red laser, and the emissions were recorded using a suitable optical filter (APC-Alexa Fluor ® 660 nm). At least 10,000 events were analyzed for each measurement.

2.5. In vitro fluorescence imaging

HeLa cells were seeded onto coverslip in 35-mm culture dishes overnight. The cells were then incubated with 10 μM complexes **1–4** for 20 h at 37 °C and 5% CO_2. For colocalization experiments, the cells were then washed with 1X PBS and stained with 50 nM Lysosome Tracker Green for 15 min. The emitted fluorescent signals of complexes and the tracker were examined with an inverted fluorescence microscope (Zeiss Axio Observer Z1, Zeiss, Germany) equipped with a UV lamp, mercury bulbs and a customized fluorescence filter (excitation wavelength=365 nm, emission wavelength=610 nm). A 63X oil immersion objective was used for imaging.

2.6. Photo-cytotoxicity assay

The PDT cytotoxicity assay was performed according to standard methods. In general, HeLa cells (3×10^3/well) were incubated in 96-well plates 24 h prior to exposure to drugs. The cells were treated with samples **1–4** in the dark overnight. Afterwards, the cells were exposure to yellow light (1–4 J/cm^2) produced from a 400 W tungsten lamp fitted with a heat-isolation filter and a 550 nm long-pass filter. The fluence rate was 6 mW/cm^2. Cell viability was determined by the MTT reduction assay at 24 h post-PDT [23]. The cell monolayers were rinsed twice with PBS and then incubated with 50 μL MTT solution (0.5 mg/mL) at 37 °C for 3 h. Then the media were removed, and 100 μL of DMSO solubilizing reagent was added and shaked for 30 min to dissolve the formed formazan crystals in living cells. The absorbance was measured at dual wavelength, 540 nm and 690 nm, on a Labsystem Multiskan microplate reader (Merck Eurolab, Switzerland). Each dosed concentration at individual light exposure was performed in quadruplicate wells for the PDT assay.

3. Results and discussion

3.1. Photophysical properties

The UV-visible absorption spectra of the four conjugates (**1–4**) were measured in various solvents ranging from the non-polar chloroform ($CHCl_3$) to the polar acetonitrile (MeCN), ethanol (EtOH) and deionised (DI) water as shown in Fig. 2. The absorption band at 288 nm was observed in all four conjugates and is assigned to the π-π* transition of the bipyridine (bpy) ligand of Ru(II) complex. The strong porphyrin Soret band is located at ~420 nm for the free-base porphyrin conjugates (**1**, **2**) and ~425 nm for the