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**ARTICLE** *in* CHEMISTRY - A EUROPEAN JOURNAL · JULY 2012

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# New Ruthenium(II) Complexes Functionalized with Coumarin Derivatives: Synthesis, Energy-Transfer-Based Sensing of Esterase, Cytotoxicity, and Imaging Studies

**Mei-Jin Li,[a, b] Keith Man-Chung Wong,[a] Changqing Yi,[c] and Vivian Wing-Wah Yam*[a]**

*Dedicated to Prof. Hubert Le Bozec on the occasion of his 60th birthday*

**Abstract:** Two new bichromophoric ruthenium(II) complexes, $[Ru(bpy)_2(bpy\text{-}CM)](PF_6)_2$ and $[Ru(bpy)_2(bpy\text{-}CM343)](PF_6)_2$ (bpy = 2,2′-bipyridine, CM = coumarin) with appended coumarin ligands have been designed and synthesized. The energy-transfer-based sensing of esterase by the complexes has been studied by using UV/Vis and luminescence spectroscopic methods. The cytotoxicity and the cellular uptake of one of the complexes have also been investigated.

**Keywords:** coumarins · energy transfer · luminescence · ruthenium

## Introduction

Recently, fluorescent sensors based on fluorescence resonance energy transfer (FRET) have attracted significant interest and are currently being exploited for various studies and applications in molecular and supramolecular photophysics,[1] biology,[2] and molecular devices.[3] Systems of particular interest are bichromophoric molecules in which a donor and an acceptor are linked by a spacer and the excited-state energy from the donor is transferred to the acceptor. Because this process is distance- and orientation-dependent, the efficiency and the rate of energy transfer can be modified by variation of the mutual distance and the orientation of the donor and acceptor moieties, which can be achieved by external perturbation on the spacer group. These kinds of systems usually show large Stokes shifts and are potentially useful for chemosensing or biosensing applications. In recent years, some ratiometric fluorescent sensors based on FRET have been developed for protease sensing.[2b,4] An enzyme-cleavable sensor based on FRET for phosphodiesterase activity was synthesized and reported by Nagano and coworkers.[2b] Large emission spectral changes upon hydrolysis of the phosphodiester group by the enzyme were observed. Due to the cleavage of the spacer by the enzyme, the FRET efficiency is decreased and such spectral changes are studied for ratiometric measurements. Another new FRET system, involving the ruthenium(II) bathophenanthroline complex acting as the acceptor, and the carbostyril derivative acting as the donor, was synthesized by Bannwarth and coworkers.[4a] The donor and acceptor could be easily introduced into the peptide moieties and such system could be employed for a protease assay. Although there have been a number of reports on the study of ruthenium(II) polypyridyl complexes,[5] it was only until quite recently that the highly luminescent ruthenium(II) complexes of polypyridyl ligands have been utilized in bioimaging[6] despite the widespread interest in their study as oxygen sensors and DNA intercalators.[7]

Our efforts have been to study the FRET processes of transition-metal complexes and to utilize the concept of FRET for the design of FRET-based sensors, which are relatively scarce in inorganic and organometallic systems, as most of the luminescent chemosensors are based on photoinduced electron transfer (PET) and photoinduced charge transfer (PCT) processes. As an extension of our previous work on the FRET studies of coumarin-appended pyridyltricarbonylrhenium(I) 2,2′-bipyridyl complexes with oligoether spacers,[8] herein we report the synthesis, photophysics, electrochemistry, energy-transfer-based sensing of esterase, cytotoxicity, and bioimaging studies of two ruthenium(II) diimine complexes appended with a coumarin moiety (Scheme 1). The complexes containing coumarin derivatives are found to give high FRET efficiency from the coumarin unit to the ruthenium(II) complex unit. Drastic electronic absorption and emission spectral changes have been observed, resulting from the modulation of the energy-transfer process upon cleavage of the ester linkage by the enzymatic

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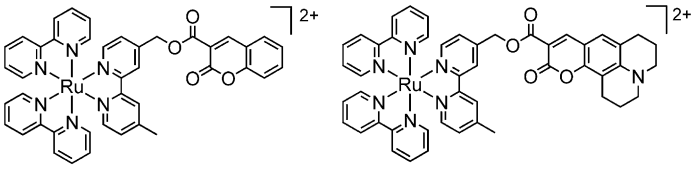
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201103025.



$[Ru(bpy)_2(bpy\text{-}CM)]^{2+}$ $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$

Scheme 1. Chemical structures of $[Ru(bpy)_2(bpy\text{-}CM)]^{2+}$ and $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ (bpy = 2,2′-bipyridine, CM = coumarin).

hydrolysis activity of esterase. The complex containing coumarin 343 has been found to exhibit only a slight cytotoxicity towards HepG2 cells and show high sensitivity toward sensing of esterase in the buffer solution and living cells, rendering it an ideal candidate for the ratiometric protease assay in a living cell.

## Experimental Section

**Materials and reagents**: Ruthenium(III) chloride hydrate, thionyl chloride, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Aldrich. 3-Carboxylic acid coumarin and 4-(2-hydroxyethyl)piperazine-1-ethane sulfonic acid (HEPES) were of analytical grade and were purchased from Lancaster Synthesis Ltd. A porcine liver esterase (PLE) suspension in $(NH_4)_2SO_4$ (3.2 M, pH 8.0) was purchased from Sigma–Aldrich and used as received. *cis*-$[Ru(bpy)_2Cl_2]\cdot 2H_2O$,[9] 4-methyl-4′-hydroxylmethyl-2,2′-bipyridine,[10] and coumarin 343 acid chloride[11] were synthesized according to literature methods.

**Synthesis of coumarin-functionalized 4,4′-dimethyl-2,2′-bipyridine (bpy-CM)**: Bpy-CM was prepared by modification of a literature method for the related compound.[12] Coumarin-3-carboxylic acid (0.23 g, 1.2 mmol) was heated to reflux with thionyl chloride (5 mL) for 2 h to give a clear solution. The excess of thionyl chloride was removed by distillation and the residue was dried in vacuum. The white solid was then dissolved in dry $CH_2Cl_2$ (5 mL), and the solution was added in a dropwise manner to a solution of 4-methyl-4′-hydroxylmethyl-2,2′-bipyridine (0.20 g, 1.0 mmol) and $Et_3N$ (0.2 mL) in dry $CH_2Cl_2$ (10 mL) at 0 °C. The mixture was heated to reflux for overnight after the addition was complete. The mixture was then cooled to room temperature and the solvent was removed under vacuum. Purification by chromatography on silica gel with $CHCl_3$ as eluent gave bpy-CM as a white solid (0.18 g, 48 %). $^1$H NMR (300 MHz, $CDCl_3$): $\delta = 8.71$ (d, $J = 4.9$ Hz, 1H; bpy), 8.63 (s, 1H; coumarin), 8.54 (d, $J = 4.9$ Hz, 1H; bpy), 8.49 (s, 1H; bpy), 8.25 (s, 1H; bpy), 7.64 (m, 2H; coumarin), 7.49 (d, $J = 4.9$ Hz, 1H; bpy), 7.36 (m, 2H; coumarin), 7.15 (d, $J = 4.9$ Hz, 1H; bpy), 5.50 (s, 2H; $CH_2O$), 2.45 ppm (s, 3H; $CH_3$); MS (EI+): *m/z*: 372 $[M]^+$.

**Synthesis of coumarin 343-functionalized 4,4′-dimethyl-2,2′-bipyridine (bpy-CM343)**: Coumarin 343 acid chloride (155 mg, 0.51 mmol) was dissolved in dry $CH_2Cl_2$ (10 mL) and the solution was added in a dropwise manner to the solution of 4-methyl-4′-hydroxylmethyl-2,2′-bipyridine (0.10 g, 0.5 mmol) and $Et_3N$ (0.75 mL) in dry $CH_2Cl_2$ (15 mL) at 0 °C. After addition, the mixture was stirred at 60 °C for 12 h. The mixture was cooled to room temperature and the solvent was removed under vacuum. Purification by chromatography on silica gel with $CHCl_3$ as eluent gave bpy-CM343 as an orange-red solid (0.15 g, 64 %). $^1$H NMR (400 MHz, $CDCl_3$): $\delta = 8.68$ (d, $J = 4.9$ Hz, 1H; bipyridyl proton at 6-position), 8.54 (d, $J = 4.9$ Hz, 1H; bipyridyl proton at 3-position), 8.41 (s, 1H; bipyridyl proton at 6′-position), 8.39 (s, 1H; coumarin 343), 8.23 (s, 1H; bipyridyl proton at 3′-position), 7.53 (d, $J = 4.9$ Hz, 1H; bipyridyl proton at 5-position), 7.14 (d, $J = 4.9$ Hz, 1H; bipyridyl at 5′-position), 6.95 (s, 1H; coumarin 343), 5.44 (s, 2H; $CH_2O$ on bpy), 3.34 (m, 4H; coumarin 343), 2.88 (t, $J = 6.2$ Hz, 2H; coumarin 343), 2.75 (t, $J = 6.2$ Hz, 2H; coumarin 343), 2.44 (s, 3H; $CH_3$ on bpy), 1.97 ppm (m, 4H; coumarin 343) MS (EI+): *m/z*: 467 $[M]^+$.

**Synthesis of $[Ru(bpy)_2(bpy\text{-}CM)](PF_6)_2$**: The complex was synthesized according to a modification of a literature procedure for the related ruthenium(II) polypyridine complexes.[9] Bpy-CM (41 mg, 0.11 mmol) was added to a solution of *cis*-$[Ru(bpy)_2Cl_2]\cdot 2H_2O$ (52 mg, 0.1 mmol) in absolute ethanol (50 mL) and the mixture was heated to reflux under $N_2$ for 5 H during which the purple-black solution turned red-orange. After removal of solvent under vacuum, the residue was dissolved in a minimum amount of water (1 mL), and the complex was precipitated by addition of a saturated aqueous $KPF_6$ solution. The desired product was obtained by filtration and subsequent recrystallization by slow diffusion of diethyl ether vapor into an acetonitrile solution of the complex afforded the product as red-orange crystals (64 mg, 60 %).$^1$H NMR (400 MHz, $CD_3CN$): $\delta = 8.74$ (s, 1H; aromatic proton), 8.70 (s, 1H; aromatic proton), 8.48 (m, 4H; aromatic protons), 8.42 (s, 1H; aromatic proton), 8.05 ( m, 4H; aromatic protons), 7.72 (m, 7H; aromatic protons), 7.54 (d, $J = 5.6$ Hz, 1H; bpy), 7.48 (d, $J = 5.6$ Hz, 1H; bpy), 7.40 (m, 6H; aromatic protons), 7.25 (d, $J = 5.6$ Hz, 1H; bpy), 5.55 (s, 2H; $CH_2O$), 2.54 ppm (s, 3H; $CH_3$); MS (FAB+): *m/z*: 931$[M-PF_6]^+$, 786 $[M-2PF_6]^+$; elemental analysis calcd (%) for $C_{42}H_{32}N_6O_4RuP_2F_{12}\cdot 1.5H_2O$ (1103): C 45.74, H 3.20, N 7.62; found: C 45.90, H 3.21, N 7.52.

**Synthesis of $[Ru(bpy)_2(bpy\text{-}CM343)](PF_6)_2$**; The complex was prepared by using a procedure similar to that for $[Ru(bpy)_2(bpy\text{-}CM)](PF_6)_2$ except bpy-CM343 was used instead of bpy-CM. Subsequent recrystallization by slow diffusion of diethyl ether vapor (51.4 mg, 0.11 mmol) into an acetonitrile solution of the complex afforded a red solid (76 mg, 65 %). $^1$H NMR (400 MHz, $CD_3CN$): $\delta = 8.73$ (d, $J = 4.9$ Hz, 1H; aromatic proton), 8.48 (m, 6H; aromatic protons), 8.05 (m, 4H; aromatic protons), 7.72 (m, 6H; aromatic protons), 7.54 (d, $J = 5.6$ Hz, 1H; bpy), 7.48 (d, $J = 5.6$ Hz, 1H; bpy), 7.40 (m, 4H; aromatic protons), 7.25 (d, $J = 5.6$ Hz, 1H; bpy), 7.03 (s, 1H; coumarin 343), 5.49 (s, 2H; $CH_2O$), 3.42 (m, 4H; coumarin 343), 2.72 (m, 4H; coumarin 343), 2.55 (s, 3H; $CH_3$), 1.94 ppm (m, 4H; coumarin 343); MS (FAB+): *m/z*: 1026 $[M-PF_6]^+$, 881 $[M-2PF_6]^+$, 441 $[M-2PF_6]^{2+}$; elemental analysis calcd for $C_{48}H_{41}N_7O_4RuP_2F_{12}\cdot H_2O$ (1189): C 48.49, H 3.64, N 8.25; found: C 48.56, H 3.58, N 8.29.

**Cell culture**: HepG2 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in RMPI 1640 medium supplemented with 10 % fetal bovine serum, 1 % penicillin, and streptomycin in a humidified 5 % $CO_2$ atmosphere.

**Cell viability assay**: The MTT assay was used to determine the viability of HepG2 cells upon treatment with $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$, as described in detail elsewhere.[13] HepG2 cells were seeded in 96-well tissue culture plates at the density of $4\times10^6$ cells per well and incubated for three days. After treatment with $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ for 24 h, the plates were washed twice with culture medium, then MTT was added and the plates were incubated for another 4 h. Cells without treatment of $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ were used as control. The relative cytotoxicity was expressed in percentage of $[OD_{sample}-OD_{blank}]/[OD_{control}-OD_{blank}]\times 100$. Data were collected from three independent experiments and expressed as the mean ± standard deviation (SD). The statistical differences were analyzed by a paired Student's *t*-test. *P* values less than 0.05 were considered to indicate statistical differences.

**Live cell confocal imaging**: HepG2 cells in growth medium ($1\times10^5$ cells mL$^{-1}$) were seeded in a 35 mm tissue culture dish and incubated at 37 °C under a 5 % $CO_2$ atmosphere for 48 h. The culture medium was removed and replaced with a mixture of medium/DMSO (99:1, v/v) containing $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ (50 μM). After incubation for 1 h, the medium was removed, and the cell layer was washed gently with PBS. The cell layer was then trypsinized and added up to a final volume of 3 mL with PBS. Imaging was performed by using a confocal microscope (Leica TCS SP5) with an excitation wavelength at $\lambda = 432$ nm. The emission was measured by using a long-pass filter at $\lambda = 455$ nm.

**Michaelis–Menten kinetics of esterase hydrolysis**: An aliquot of porcine liver esterase (PLE, 6 μL) was added into a cuvette containing HEPES buffer (2.4 mL, 0.05 M, pH 7.5, 0.1 M KCl) and various concentrations

(0.1–20 μM) of the complex. The cuvette was shaken vigorously and the emission intensity at $\lambda = 488$ nm (excitation at $\lambda = 445$ nm) was monitored for 5 min at 25 °C. The rate of increase in emission intensity between 100 and 200 s was determined and converted into μM min$^{-1}$ of coumarin 343 by using a standard curve. The rate of increase in concentration of coumarin 343 was plotted as a function of substrate concentration and a theoretical fit according to a Michaelis–Menten model was performed [Eq. (1)].

$$V = \frac{V_{max}[S]}{K_M + [S]} \quad (1)$$

In Equation (1) $V$ is the rate of reaction, $V_{max}$ is the maximum reaction rate, [S] is the concentration of the substrate, $K_M$ is the Michaelis constant, an indicator of the affinity that an enzyme has for a given substrate.

**Physical measurements and instrumentation**: Electronic absorption spectra were recorded on a Hewlett–Packard 8452A diode array spectrophotometer. Steady-state emission was recorded on a Spex Fluorolog-2 Model F111 fluorescence spectrophotometer. $^1$H NMR spectra were recorded on a Bruker DPX-300 or Bruker DPX-400 Fourier transform NMR spectrometer with chemical shifts were reported relative to tetramethylsilane. FAB+ and EI+ mass spectra were recorded on a Finnigan MAT95 mass spectrometer. Elemental analysis of the complexes was performed on a Carlo Erba 1106 elemental analyzer at the Institute of Chemistry of the Chinese Academy of Sciences in Beijing.

Luminescence quantum yields were measured by the optical dilute method reported by Demas and Crosby.[15] A degassed aqueous solution of $[Ru(bpy)_3]Cl_2$ ($\varphi = 0.042$, excitation wavelength at $\lambda = 436$ nm) was used as the reference.[16] All solutions for photophysical studies were degassed on a high-vacuum line in a two-compartment cell consisting of a 10 mL Pyrex bulb and a 1 cm path length quartz cuvette and sealed from the atmosphere by a Bibby Rotaflo HP6 Teflon stopper. The solutions were subject to no less than four freeze-pump-thaw cycles.

Energy transfer efficiency ($\eta_{ET}$) was determined by using the Equation (2), in which $I_D$ and $I_{DA}$ are the donor emission intensities with and without the acceptor, respectively; and $\varphi_D$ and $\varphi_{DA}$ are the donor emission quantum yields with and without the acceptor, respectively.

$$\eta_{ET} = 1-(I_D/I_{DA}) \text{ or } 1-(\varphi_D/\varphi_{DA}) \quad (2)$$

## Results and Discussion

**Synthesis and characterization**: Conversion of coumarin 3-carboxylic acid to coumarin 3-carboxylic chloride was achieved by the reaction of thionyl chloride under heating to reflux conditions for 5 h. Reaction of 4-methyl-4′-hydroxylmethyl-2,2′-bipyridine[10] with coumarin 3-carboxylic chloride or coumarin 343 acid chloride[11] afforded the ligands bpy-CM and bpy-CM343, respectively. The ruthenium(II) complexes were synthesized by reacting the ligand bpy-CM or bpy-CM343 with *cis*-$[Ru(bpy)_2Cl_2]\cdot 2H_2O$ in absolute ethanol under heating to reflux conditions under $N_2$ for overnight. Subsequent metathesis to the $PF_6^-$ salts was accomplished by addition of a saturated aqueous solution of $KPF_6$ to the corresponding complex chlorides. The identity of the ligands was confirmed by satisfactory $^1$H NMR spectroscopy and EI mass spectrometry, whereas those of the complexes were confirmed by satisfactory elemental analyses, $^1$H NMR spectroscopy, and FAB mass spectrometry.

**Photophysical properties**: The electronic absorption spectrum of the complex $[Ru(bpy)_2(bpy\text{-}CM)](PF_6)_2$ in $CH_3CN$ or in HEPES buffer (0.05 M, pH 7.5, 0.1 M KCl) shows an intense band at $\lambda \approx 290$ nm and a less intense bands at approximately $\lambda \approx 420$–460 nm. The higher energy absorption band is ascribed to the $\pi\rightarrow\pi^*$ intraligand (IL) transition of the bipyridine ligands and coumarin, whereas the lower energy absorption band is tentatively assigned as the [dπ(Ru)→π*-(bpy-CM)] metal-to-ligand charge transfer (MLCT) transition. For $[Ru(bpy)_2(bpy\text{-}CM343)](PF_6)_2$, the electronic absorption spectrum shows an intense absorption band at $\lambda \approx 280$ nm and a less intense band at $\lambda \approx 440$ nm. The higher energy absorption band is assigned as IL $\pi\rightarrow\pi^*$ transition of the bipyridine ligands. In view of the fact that the free ligand bpy-CM343 also shows an absorption band at $\lambda = 440$ nm, the lower energy absorption band with higher extinction coefficient ($\varepsilon = 43\,590$) is accordingly assigned as a mixture of the IL transitions of coumarin 343 and the [dπ(Ru)→π*(bpy)] MLCT transition. Similar assignments for the related ruthenium(II) diimine systems have also been reported in the literature.[5] The electronic absorption data of the complexes are summarized in Table 1.

Upon excitation at the absorption wavelength of coumarin and courmarin 343, $\lambda = 300$ and 430 nm, respectively, both $[Ru(bpy)_2(bpy\text{-}CM)]^{2+}$ and $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ are found to exhibit intense orange emission at $\lambda \approx 620$ nm in MeCN or in HEPES buffer (0.05 M, pH 7.5, 0.1 M KCl) at room temperature. The origin of such an orange emission is attributed to the typical triplet [dπ(Ru)→π*(bpy)] $^3$MLCT excited state (Figure 1). The emission quantum yields of $[Ru(bpy)_2(bpy\text{-}CM)]^{2+}$ and $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ in HEPES buffer (0.05 M, pH 7.5, 0.1 M KCl) at room temperature are $0.96\times10^{-2}$ and $1.53\times10^{-2}$, respectively. The excitation spectra of $[Ru(bpy)_2(bpy\text{-}CM)]^{2+}$ and $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ in HEPES buffer (0.05 M, pH 7.5, 0.1 M KCl) are depicted in Figures S1 and S2 in the Supporting Information, respectively. It is interesting to note that an additional emission band at $\lambda = 485$ nm assignable to the intraligand emission from coumarin 343 is observed in $[Ru(bpy)_2(bpy$-

Table 1. Electronic absorption and electrochemical data of the $Ru^{II}$ complexes in $CH_3CN$.

| Complex | $\lambda_{abs}$ [nm] ($\varepsilon$ [dm$^3$mol$^{-1}$cm$^{-1}$]) | Oxidation[a] $E_{1/2}$ [V] versus SCE[b] ($\Delta E_p$ [mV])[c] | Reduction[a] $E_{1/2}$ [V] versus SCE[b] ($\Delta E_p$ [mV])[c] |
|---|---|---|---|
| $[Ru(bpy)_2(bpy\text{-}CM)](PF_6)_2$ | 246 (22350), 255 (20560), 288 (80190), 330 (14310), 420 (9440), 454 (12730) | +1.23 (59) | −1.20,[d] −1.39 (59), −1.59 (60), −1.85 (110) |
| $[Ru(bpy)_2(bpy\text{-}CM343)](PF_6)_2$ | 246 (29660), 254 (28020), 287 (80720), 325 (12910), 445 (43590) | +0.91 (65), +1.22 (57) | −1.41 (60), −1.61 (80), −1.85 (83) |

[a] In acetonitrile (0.1 M $n$Bu$_4$NClO$_4$) at 298 K, working electrode, glassy carbon; scan rate, 100 mVs$^{-1}$. [b] $E_{1/2} = (E_{pa}+E_{pc})/2$; $E_{pa}$ and $E_{pc}$ are peak anodic and peak cathodic potentials, respectively. [c] $\Delta E_p = E_{pa} - E_{pc}$. [d] Irreversible wave.

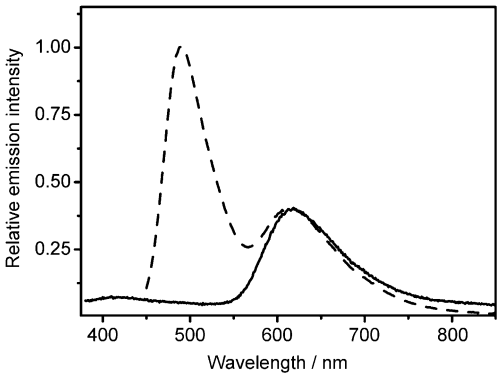



Figure 1. Emission spectra of $[Ru(bpy)_2(bpy\text{-}CM)](PF_6)_2$ (——) and $[Ru(bpy)_2(bpy\text{-}CM343)](PF_6)_2$ (-----) with excitation wavelength at $\lambda = 300$ and 430 nm, respectively, in HEPES buffer (0.05 M, pH 7.5, 0.1 M KCl) at 298 K.

CM343)]$(PF_6)_2$. On the other hand, no observable or very weak emission from the organic coumarin fluorophore is found for $[Ru(bpy)_2(bpy\text{-}CM)](PF_6)_2$ (Figure 1), even with the excitation wavelength at the coumarin absorption band at $\lambda = 300$ nm. The absence of the coumarin emission is suggestive of an efficient energy transfer from the coumarin donor to the ruthenium(II) complex acceptor in $[Ru(bpy)_2(bpy\text{-}CM)](PF_6)_2$. Although a quantitative energy-transfer efficiency from the coumarin to the ruthenium(II) moiety in $[Ru(bpy)_2(bpy\text{-}CM)]^{2+}$ cannot be determined due to the observation of very weak to negligible emission from the coumarin fluorophore, the energy-transfer efficiency of the unity is expected from this observation. On the contrary, the observation of the characteristic emission of coumarin 343 in $[Ru(bpy)_2(bpy\text{-}CM343)](PF_6)_2$ is attributed to the less efficient energy transfer from coumarin 343 to the ruthenium(II) moiety, with an energy-transfer efficiency of about 95%, probably due to the less effective overlap between the coumarin 343 emission and the $^1$MLCT absorption band of the ruthenium(II) complex.

**Electrochemical properties**: The ruthenium(II) complexes $[Ru(bpy)_2(bpy\text{-}CM)]^{2+}$ and $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ show typical oxidation couples at approximately +1.2 V versus SCE (Table 1), which are assigned as the $Ru^{II}$ to $Ru^{III}$ oxidation, on the basis of similar oxidation reported in other related ruthenium(II) diimine complexes.[17] An additional quasi-reversible couple at approximately +0.9 V versus SCE is observed in $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$. Such a quasi-reversible couple is tentatively assigned as the oxidation of the amino group on coumarin 343. Both complexes show three quasi-reversible reduction couples at about −1.40, −1.60, and −1.85 V versus SCE, and an additional irreversible cathodic wave at −1.20 V versus SCE is observed in $[Ru(bpy)_2(bpy\text{-}CM)]^{2+}$. For $[Ru(bpy)_2(bpy\text{-}CM)]^{2+}$, the cathodic wave at −1.20 V and the reduction couple at −1.40 V are tentatively assigned to the reductions of coumarin and bipyridine ligand on the bpy-CM ligand in view of the observation of similar reductions in the cyclic voltammogram of the free ligand bpy-CM. On the other hand, the reduction couples at about −1.60 and −1.85 V are attributed to the successive reductions of the two bipyridine ligands, similar to that observed in the related ruthenium(II) diimine complexes.[17] Similarly, the first reduction couple at −1.40 V is assigned to ligand-centered reductions of bpy-CM343, whereas the second and the third reduction couples at about −1.60 and −1.85 V are ascribed to the successive reductions of the two bipyridine ligands in $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$. The occurrence of the ligand-centered reduction of bpy-CM and bpy-CM343 at a less negative potential is probably due to the presence of an electron-withdrawing ester group on the bipyridine, resulting in the stabilization of the $\pi^*$ orbital of the bpy-CM and bpy-CM343 ligands.

**Enzyme-cleavable sensing for esterase activity**: The sensing behaviors of the ruthenium(II) complexes for esterase activity have been investigated by electronic absorption and emission spectroscopic methods. The electronic absorption spectral changes of $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ after treatment of PLE are studied in HEPES buffer (0.05 M, pH 7.5, 0.1 M KCl). The low-energy absorption band is found to shift to the blue slightly from $\lambda = 455$ to approximately 435 nm, corresponding to the absorption band of free coumarin 343. The absorbance at $\lambda = 455$ nm is decreased by approximately 16% upon treatment with PLE over one hour. Similar observations have also been reported in other related systems.[4e] Two well-defined isosbestic points at $\lambda \approx 330$ and 430 nm are observed. Time-dependent studies on the electronic absorption spectral changes for $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ over one hour upon treatment with PLE have been performed (Figure 2) and the change in the absorbance as a function of time reveals an exponential decay trace (inset of Figure 2). It is noteworthy that $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ has been hydrolyzed by porcine liver esterase in HEPES buffer, which leads to the large changes in the spectroscopic properties. In contrast, no hydrolysis by PLE is observed in the complex $[Ru(bpy)_2(bpy\text{-}CM)]^{2+}$.

As the observation of emission at $\lambda = 485$ and 620 nm in $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ is suggestive for energy transfer

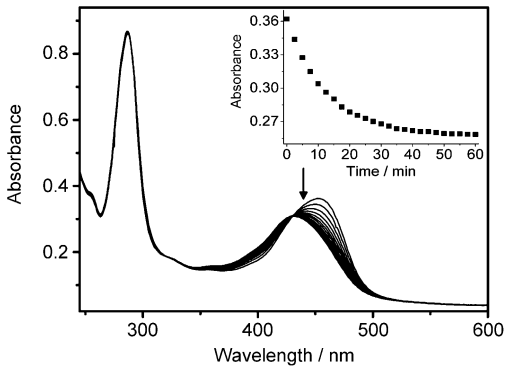



Figure 2. Electronic absorption spectral changes of $[Ru(bpy)_2(bpy\text{-}CM343)](PF_6)_2$ upon treatment of PLE over one hour. The inset shows the change in absorbance at $\lambda = 455$ nm as a function of time.

from the coumarin 343 donor to the ruthenium(II) acceptor, such an energy-transfer process is anticipated to be modulated upon the cleavage of the ester group by the hydrolysis of PLE activity. Upon addition of PLE to a solution of [Ru(bpy)$_2$(bpy-CM343)]$^{2+}$ in HEPES buffer, the emission spectrum with the excitation at the isosbestic wavelength of 430 nm exhibits a drastic emission enhancement at $\lambda =$ 485 nm, whereas the characteristic emission of the ruthenium(II) luminophore at $\lambda = 620$ nm is relatively weak, as shown in Figure 3. The principle of operation of the porcine

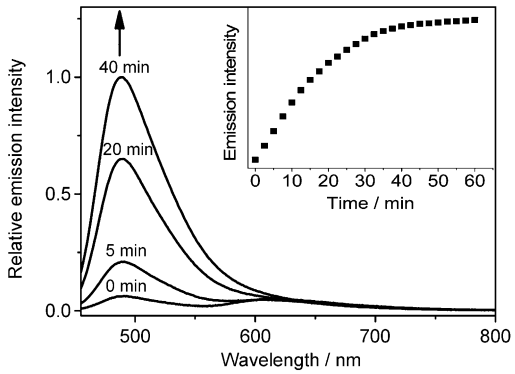
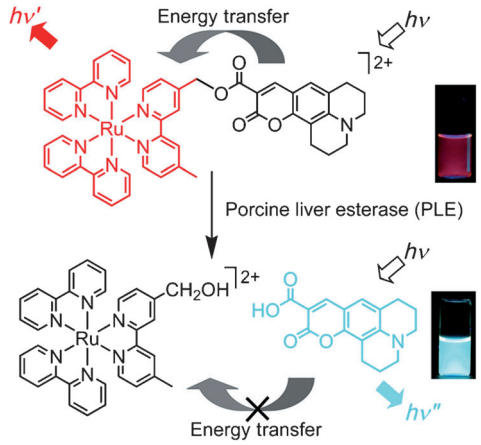



Figure 3. Emission spectral changes of [Ru(bpy)$_2$(bpy-CM343)](PF$_6$)$_2$ upon treatment with PLE over one hour. The inset shows the change in emission intensity at $\lambda = 485$ nm as a function of time.

liver esterase sensor is shown in Scheme 2. Upon addition of PLE, [Ru(bpy)$_2$(bpy-CM343)]$^{2+}$ would be hydrolyzed into the corresponding components [Ru(bpy)$_2$(bpy-CH$_2$OH)]$^{2+}$ and CM343-COOH. The luminescence quantum yields of the two units, [Ru(bpy)$_2$(bpy-CH$_2$OH)]$^{2+}$ and CM343-COOH, in HEPES buffer have been determined to be $3.77\times10^{-2}$ and 0.2848, respectively. No direct conclusion could be inferred from a comparison of the luminescence quantum yield of [Ru(bpy)$_2$(bpy-CH$_2$OH)]$^{2+}$ with that of [Ru(bpy)$_2$(bpy-CM343)]$^{2+}$, because the luminescence quantum yield of the former is even larger than that of the later. This may probably be due to the rather large difference in their structures that leads to a difference in the luminescence properties of [Ru(bpy)$_2$(bpy-CM343)]$^{2+}$ and [Ru(bpy)$_2$(bpy-CH$_2$OH)]$^{2+}$. In view of the fact that the energy-transfer efficiency is strongly dependent on the distance between the donor and the acceptor, hydrolysis of the ester group on the bpy-CM343 ligand of [Ru(bpy)$_2$(bpy-CM343)]$^{2+}$ by PLE would separate the donor and the acceptor far apart and hence lead to the blocking of the energy-transfer process. As a result, the emission is mainly attributed to the fluorescence of coumarin 343 at $\lambda =$ 485 nm, whereas the emission from the ruthenium(II) luminophore at $\lambda = 620$ nm is relatively weak. As the excitation was made at the isosbestic wavelength of the electronic absorption spectral changes (Figure 2), the change of emission intensity due to a change in the absorbance could be minimized and the increase of the intensity for coumarin 343 is due only to the suppression of the energy-transfer process. Although a decrease in the emission intensity of the ruthenium(II) luminophore at $\lambda = 620$ nm is anticipated due to the suppression of the energy-transfer process, the observation of an apparently less-affected emission intensity of the ruthenium(II) luminophore at $\lambda = 620$ nm may not be unreasonable given the influence of the emission tail from the very intense coumarin 343 emission that would mask the intensity of the 620 nm band as well as the higher luminescence quantum yield of the hydrolyzed product, [Ru(bpy)$_2$(bpy-CH$_2$OH)]$^{2+}$ ($3.77\times10^{-2}$), than that of [Ru(bpy)$_2$(bpy-CM343)]$^{2+}$ ($1.53\times10^{-2}$). Although photoinduced electron transfer may be thermodynamically allowed based on the electrochemical data, such a process is not favored in view of the presence of the insulating methylene group between the bipyridine and the coumarin 343 moieties. Time-dependent studies on the emission spectral changes at $\lambda =$ 485 nm as a function of time after treatment with PLE over one hour have been performed (inset of Figure 3). [Ru(bpy)$_2$(bpy-CM343)]$^{2+}$ exhibits a large emission spectral change, in which the main emission maximum is shifted from 620 to 485 nm, together with a drastic emission color change after the hydrolysis of the ester group by the enzyme esterase. The kinetics of the PLE activity on [Ru(bpy)$_2$(bpy-CM343)]$^{2+}$ has been probed spectroscopically. From the Michaelis–Menten plot shown in Figure 4, the Michaelis constant ($K_M$) and the maximum reaction rate ($V_{max}$) are determined to be 1.70 μM and 0.037 μM min$^{-1}$, respectively.




Scheme 2. The principle of operation of the porcine liver esterase sensor based on the FRET mechanism and photographs showing the emission color change of the solution before and after addition of esterase.

**Cytotoxicity studies**: An MTT assay has been employed to measure the metabolic activity of mitochondria in the cells based on the principle that living cells are capable of reducing the lightly colored tetrazolium salt into an intense colored formazan derivative.[13c] Figure 5 shows the viability of HepG2 cells upon treatment with [Ru(bpy)$_2$(bpy-CM343)]$^{2+}$ for 24 h. The results reveal that [Ru(bpy)$_2$(bpy-CM343)]$^{2+}$ exhibits only a slight cytotoxicity towards HepG2 cells. A similar viability is also observed in four times higher concentration of the ruthenium(II) complex for the imaging study.

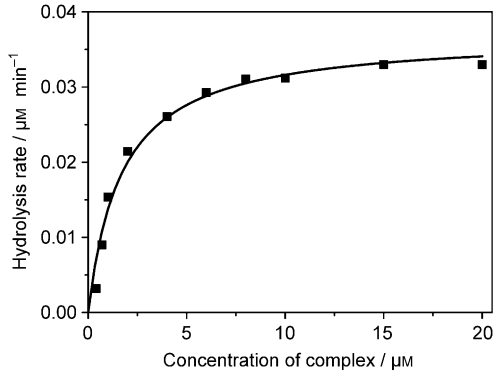



Figure 4. Michaelis–Menten plot of $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ hydrolysis by treatment of PLE.

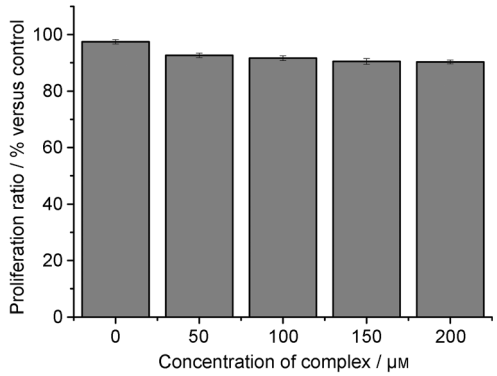



Figure 5. Viability of HepG2 cells upon treatment with $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ for 24 h.

**Sensing of esterase in living cells**: HepG2 cells are exposed to $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ at a concentration of 50 μM for one hour, in which time blue emission has been observed in the living HepG2 cells (Figures 6A and C), whereas red emission has been found in the dead cells or cell debris (Figures 6A and D). The bright-field morphology of these examined cells is shown in Figure 6B. The distribution of the complex in the cytoplasm is not even, but localized in the pronuclear region. However, the nuclei show much weaker emission, indicative of negligible nuclear uptake of the complex. From the image, it is likely that the complex binds to the hydrophobic organelles such as the Golgi apparatus, the endoplasmic reticulum, and the mitochondria.[18] All the intracellular luminescence images are readily observable without the need of prior washing to remove $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ in the medium. In fact, the complex is rapidly taken up and reacted in the living cells. It is believed that the esterase in the living cells would react with $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$ and cause a change in the luminescence from red to blue. Therefore, the need for an extensive washing step before imaging is not necessary due to the unique property of this $Ru^{II}$ complex. Moreover, the omission of the washing step allows the internalization process of the dye into the living cells to be visualized by time-lapsed microscopy.

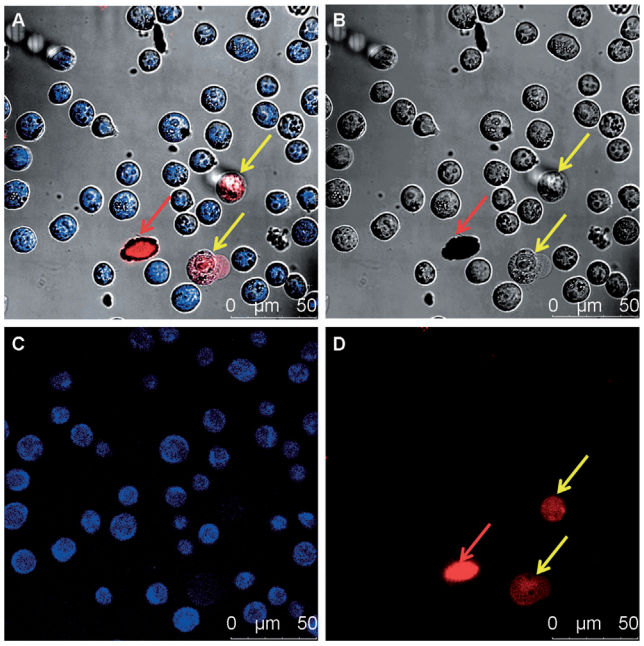



Figure 6. Images of confocal laser scanning microscopy of HepG2 cells in the presence of $[Ru(bpy)_2(bpy\text{-}CM343)]^{2+}$: A) Merged image; B) bright-field image; C and D) fluorescence images. Yellow arrows indicate the dead cells, whereas red arrow indicates cell debris.

## Conclusion

Two new bichromophoric ruthenium(II) complexes with appended coumarin ligands have been designed and synthesized. The energy-transfer process from the coumarin donor to the ruthenium(II) acceptor has been observed. The cleavage of the ester linkage in the complexes by esterase together with its associated spectroscopic changes has been studied. The electronic absorption and emission spectra exhibited a large spectral change, resulting from the reduced FRET efficiency after the hydrolysis of the ester group by the enzyme due to the cleavage of the spacer. The $Ru^{II}$ complex containing the coumarin 343 derivative is found to exhibit only a slight cytotoxicity towards HepG2 cells, and it is easily taken up in the living cells to show obvious response to esterase as shown in the imaging study.

## Acknowledgements

V.W.-W.Y. acknowledges support from the University of Hong Kong under the Distinguished Research Achievement Award Scheme. This work has also been supported by a Collaborative Research Fund (CRF) grant (HKUST2/CRF/10) and a General Research Fund (GRF) grant (HKU 7064/11P) from the Research Grants Council of Hong Kong Special Administrative Region, China. M.-J.L. acknowledges the National Natural Science Foundation of China (NSFC No. 20801014).

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Received: September 27, 2011
Published online: June 12, 2012

  