

Methods to Explore Cellular Uptake of Ruthenium Complexes

Cindy A. Puckett and Jacqueline K. Barton*

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125

Received October 30, 2006; E-mail: jkbarton@caltech.edu

The cellular uptake characteristics of a small molecule are critical to its application as a therapeutic or diagnostic agent. However, our understanding of the chemical rules governing uptake is rudimentary.^{1,2} Although transition metal complexes have increasingly been applied for biological applications,^{3–5} their uptake properties are even less well developed. Here, we exploit flow cytometry to provide statistics on the uptake of ruthenium complexes into HeLa cells. These ruthenium complexes, owing to their facile synthesis, stability, and luminescence, provide a route to compare and contrast factors governing cellular uptake.

A series of dipyrrophenazine (dppz) complexes of Ru(II) were synthesized for systematic comparison.^{6–8} Substituting the ancillary ligands on the dppz complex permits variation in the overall complex charge, size, and hydrophobicity (Figure 1).

Furthermore, since these dppz complexes all act as molecular light switches, showing minimal luminescence in aqueous solution and intense luminescence when bound to DNA or otherwise protected from water, they provide a sensitive cellular probe (Table 1).^{9–11}

HeLa cells were prepared for flow cytometry analysis after incubation with the Ru complexes at various concentrations and times.¹² Flow cytometry was performed on a BD FACS Aria using ~20 000 cells per sample. The ruthenium complexes were excited at 488 nm, with the emission observed at 600–620 nm. Live cells were distinguished by their low To-Pro-3 emission.

Figure 2 illustrates results of the flow cytometry. Cells not treated with complex exhibit some background luminescence. Incubation with 10 μ M Ru(bpy)₂dppz²⁺ or Ru(phen)₂dppz²⁺ for 2 h causes only a small change in the luminescence profile. When cells are incubated with 10 μ M Ru(DIP)₂dppz²⁺, however, the luminescence intensity of the cell population increases dramatically.

Uptake for the different Ru complexes may be compared based upon the mean luminescence intensity of the cell population (Table 2). Below 1 μ M, Ru(DIP)₂dppz²⁺ is taken up appreciably above background. At higher concentrations, Ru(bpy)₂dppz²⁺, Ru(CO₂-Et-bpy)₂dppz²⁺, and Ru(phen)₂dppz²⁺ are taken up to some extent, but even at 20 μ M Ru, little luminescence is evident for Ru-(mcbpy)₂dppz.¹³ Washing with buffer reduces luminescence by 20–50%, suggesting that, while some Ru is nonspecifically adhered to the surface or rapidly exported, the bulk of Ru remains.

That the complexes are actually transported into the cellular interior rather than associating solely at the membrane surface is evident by confocal microscopy (Figure 3).¹⁴ For Ru(DIP)₂dppz²⁺, intense luminescence in the interior is apparent within 2 h. For Ru(phen)₂dppz²⁺ and Ru(bpy)₂dppz²⁺, microscopy experiments also show uptake into the cellular interior but, consistent with the flow cytometry data, on a slower time scale (≥ 4 h for phen). For all complexes, greatest luminescence is evident in the cytoplasm, likely associated with the mitochondria and endoplasmic reticulum based upon costaining experiments with organelle-specific dyes;¹⁵ without protection from water through macromolecular binding, Ru quenching in the cytosol is expected.

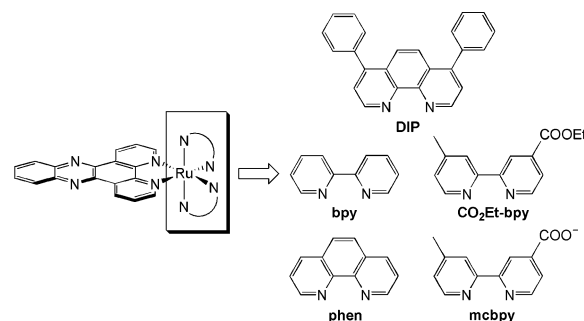


Figure 1. Dipyrrophenazine complexes of Ru(II).

Table 1. Characteristics of Ru Complexes

ancillary ligand of RuL ₂ dppz	relative emission intensity in CH ₃ CN ^a	relative emission intensity w/DNA ^{a,b}	octanol/H ₂ O partition coefficient (log <i>P</i>) ^c	diameter (Å) ^d
bpy	1.0	1.0	−2.50	16.2
CO ₂ Et-bpy	1.8	1.1	−0.76	20.4
mcbpy	1.2	0.6	−0.43	18.2
phen	1.2	2.3	−1.48	16.2
DIP	2.7	2.7	1.30	20.4

^a Excited at 488 nm; integrated emission at 600–620 nm; 10 μ M Ru was used, except for Ru(DIP)₂dppz²⁺ in Tris buffer, where a lower concentration was used due to poor solubility; emission values were scaled accordingly. ^b Luminescence values with DNA were obtained at saturation. ^c Cl[−] salt. ^d Diameters were estimated using Titan.

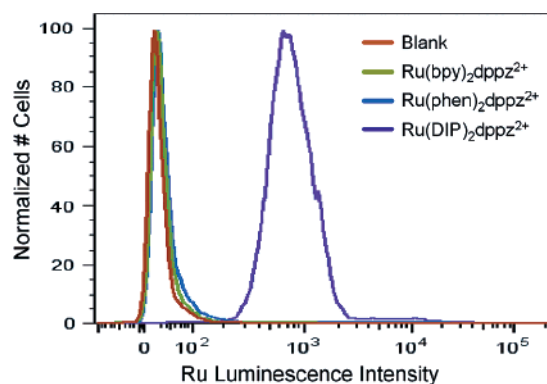


Figure 2. Flow cytometry analysis of HeLa cells incubated with 10 μ M ruthenium complex for 2 h. Luminescence data were obtained by excitation at 488 nm with emission at 600–620 nm using a light scatter gate to exclude debris and To-Pro-3 (exciting at 633 nm and observing at 650–670 nm) to exclude dead cells.

Interestingly, significantly less luminescence is apparent in the nucleus. Quantitation by line plots (Figure 3) does show nuclear uptake but of diminished intensity in the nucleus compared to other regions.¹⁶ Flow cytometry experiments on nuclear preparations isolated after incubation of cells with Ru(DIP)₂dppz²⁺ provide consistent evidence of Ru uptake (Supporting Information).

These data establish that the ruthenium complexes are indeed taken up inside HeLa cells. If the complexes are entering the cell

Table 2. Mean Luminescence Intensity of HeLa Cells Incubated with Ruthenium Complex by Flow Cytometry^a

concn (μ M)	Ancillary Ligands of RuL ₂ dppz				
	bpy	CO ₂ Et-bpy	mcbpy	phen	DIP
0.5	n.d.	n.d.	n.d.	n.d.	60
1	n.d.	n.d.	n.d.	n.d.	99
5	38	45	20	52	597
10 ^b	38	45	21	58	974 (571)
20 ^b	48 (27)	51 (29)	26 (19)	111 (50)	n.d.

^a Cells were incubated with ruthenium complex for 2 h at ambient temperature. Ruthenium complexes were excited at 488 nm, with emission observed at 600–620 nm. The mean luminescence intensity of cells not treated with complex is 23. Data are an average of two independent experiments, and data not determined are indicated by n.d. ^b Samples washed after incubation are shown in parentheses.

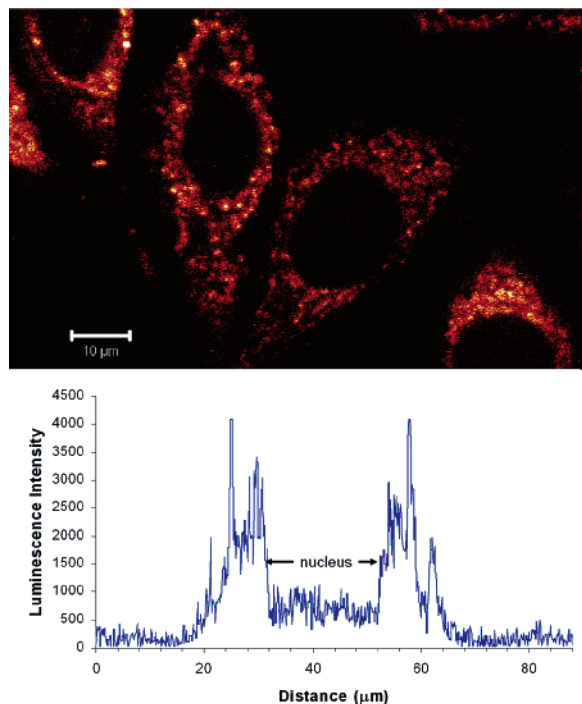


Figure 3. Confocal microscopy of HeLa cells with Ru(DIP)₂dppz²⁺. (Top) Microscopy after incubation with 5 μ M Ru for 2 h at 37 °C. Excitation wavelength = 488 nm. (Bottom) Intensity profile of ruthenium luminescence across a HeLa cell after incubation for 12 h with 10 μ M Ru.

by passive diffusion, one would predict that neutral charge, smaller size, and greater hydrophobicity should aid uptake. Here, however, cellular uptake appears to be facilitated by the lipophilic DIP ligand, despite the larger size of the complex. It is surprising that the large expanse of the DIP complex does not limit its uptake. Also of interest, changing the overall charge from +2 to neutral does not improve uptake, based upon the low luminescence results for Ru(mcbpy)₂dppz.¹⁷

There have been few systematic studies on the cellular uptake of transition metal complexes reported.^{18–20} Our results are in agreement with studies on cisplatin analogues, where the complexes with the greatest lipophilicity exhibit the highest uptake; note that for the Pt complexes, all were hydrophilic, with octanol/water partition coefficients of <1.²⁰ Importantly, these data also establish

that the Ru complexes are stable to the intracellular environment; no loss of luminescence is evident, as would be expected based upon changes in complex coordination.

Flow cytometry, traditionally used to examine organic fluorophores, thus provides an opportunity to examine the cellular uptake of transition metal complexes. Ruthenium analogues in particular can be readily tested without special instrumentation or complicated synthesis. Statistics on thousands of cells of varied cell type and using a range of metal complexes can be generated to provide a powerful complement in the design of metal complexes for biological application.

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Supporting Information Available: Flow cytometry for cell nuclei. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) DeVito, S. C. In *Handbook of Property Estimation Methods for Chemicals: Environmental and Health Sciences*; Boethling, R. S.; Mackay, D., Eds.; CRC Press: Boca Raton, FL, 2000; pp 261–278.
- (2) Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug Delivery Rev.* **2001**, *46*, 3.
- (3) Zhang, C. X.; Lippard, S. J. *Curr. Opin. Chem. Biol.* **2003**, *7*, 481.
- (4) Boerner, L. J. K.; Zaleski, J. M. *Curr. Opin. Chem. Biol.* **2005**, *9*, 135.
- (5) (a) Brunner, J.; Barton, J. K. *Biochemistry* **2006**, *45*, 12295. (b) Hart, J. R.; Glebov, O.; Ernst, R. J.; Kirsch, I. L.; Barton, J. K. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 15359.
- (6) Dickeson, J. E.; Summers, L. A. *Aust. J. Chem.* **1970**, *23*, 1023.
- (7) Ru(phen)₂Cl₂ and Ru(DIP)₂Cl₂ were synthesized in analogous fashion to Ru(bpy)₂Cl₂ described in Sullivan, B. P.; Salmon, D. J.; Meyer, T. J. *Inorg. Chem.* **1978**, *17*, 3334. The dppz ligand was then added by refluxing in ethanol–water for >3 h.
- (8) Ru(CO₂Et-bpy)₂Cl₂ was synthesized using a modification of Leasure, R. M.; Ou, W.; Moss, J. A.; Linton, R. W.; Meyer, T. J. *J. Chem. Mater.* **1996**, *8*, 264. DME–ethanol (2:1) was used as the reaction solvent. The dppz ligand was added by refluxing in EtOH for 24 h. Ru(mcbpy)₂dppz was formed by hydrolysis of the ester.
- (9) (a) Friedman, A. E.; Chambron, J.-C.; Sauvage, J.-P.; Turro, N. J.; Barton, J. K. *J. Am. Chem. Soc.* **1990**, *112*, 4960. (b) Jenkins, Y.; Friedman, A. E.; Turro, N. J.; Barton, J. K. *Biochemistry* **1992**, *31*, 10809.
- (10) Olofsson, J.; Önfelt, B.; Lincoln, P. J. *Phys. Chem. A* **2004**, *108*, 4391.
- (11) Ardhammer, M.; Lincoln, P.; Nordén, B. *J. Phys. Chem. B* **2001**, *105*, 11363.
- (12) HeLa cells were detached from monolayer culture with EDTA, resuspended in Hank's balanced salt solution supplemented with bovine serum albumin fraction 5, and diluted to 1 × 10⁶ cells/mL. The ruthenium complexes were added to the cell suspensions at concentrations of 0.5–10 μ M and incubated for 2 h at ambient temperature. Dead cells were stained with 1 μ M To-Pro-3 (Molecular Probes), which enters cells having compromised membranes.
- (13) The low luminescence from Ru(mcbpy)₂dppz may be in part due to poor nucleic acid binding by the neutral complex.
- (14) Confocal microscopy of cells incubated with a Ru dimer has been reported. See: Önfelt, B.; Gostring, L.; Lincoln, P.; Nordén, B.; Önfelt, A. *Mutagenesis* **2002**, *17*, 317.
- (15) Haugland, R. P. In *Handbook of Fluorescent Probes and Research Products*, 9th ed.; Gregory, J., Spence, M., Eds.; Molecular Probes: Eugene, OR, 2002; pp 473–488 and 496–502.
- (16) After incubation of HeLa cells for 2 h with 5 μ M Ru(DIP)₂dppz²⁺, line plot quantitation shows an average of 11% luminescence in the nucleus compared to cytoplasm; for 10 μ M Ru after 12 h, as in Figure 3 (bottom), the ratio is ~30%. Some contribution to fluorescence in the nucleus could arise from cellular autofluorescence.
- (17) The lower luminescence of Ru(mcbpy)₂dppz with nucleic acids contributes to its relatively poor luminescence in cells but cannot fully account for it.
- (18) Jonas, S. K.; Riley, P. A. *Cell Biochem. Funct.* **1991**, *9*, 245.
- (19) Kalayda, G. V.; Fakih, S.; Bertram, H.; Ludwig, T.; Oberleithner, H.; Krebs, B.; Reedijk, J. J. *Inorg. Biochem.* **2006**, *100*, 1332.
- (20) Ghezzi, A.; Aceto, M.; Cassino, C.; Gabano, E.; Osella, D. *J. Inorg. Biochem.* **2004**, *98*, 73.

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