Observation of the selective staining of chromosomal DNA in dividing cells using a luminescent terbium(III) complex†

Ga-Lai Law,[a] Cornelia Man,[b] David Parker*[a] and James W. Walton[a]

Received (in Cambridge, UK) 24th November 2009, Accepted 16th January 2010
First published as an Advance Article on the web 2nd February 2010
DOI: 10.1039/b924679g

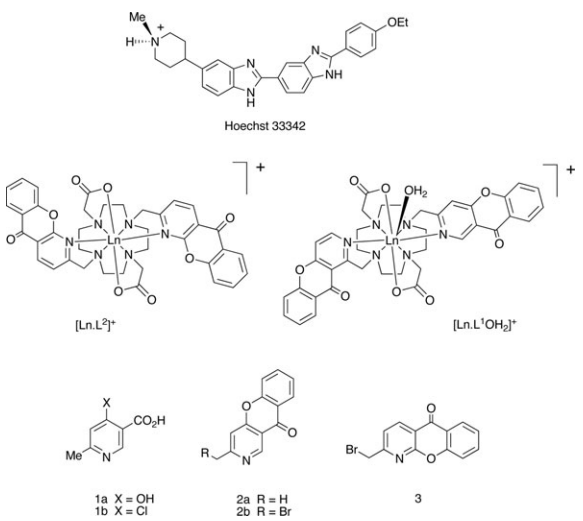
The emission intensity of a monocationic Tb or Eu(III) complex of a bis(1-azaxanthone) ligand is enhanced in the presence of serum albumin; the lanthanide complex stains dividing cells, allowing visualisation of mitotic chromosomes.

Non-invasive live cell imaging is the key to obtain information on cell division (mitosis). In current practice, membrane-permeable organic dyes that specifically bind DNA are used to visualise chromosomes in live cells.[1] A typical example is the fluorescent cationic dye, Hoechst 33342 (λ_{exc} 350 nm, λ_{em} 461 nm). It permeates the outer and nuclear membranes of cells non-selectively and exhibits a fluorescence enhancement when bound to nuclear DNA. Care needs to be exercised in its usage as higher concentrations and direct UV-phototoxicity can lead to cellular damage.[2,3]

Out of all the different phases in the cell cycle, the 'M' phase is most sensitive and has in-built stringent controls for phototoxicity. Thus, a dividing cell will rapidly raise 'red flags' when it is damaged, resulting in the reversal to the 'G-2' state or the activation of the spindle assembly checkpoint. The consequence of checkpoint activation is a pause or a full arrest in mitotic progression. The cell may also return to a 'G-2' like state and, in more extreme cases, undergo apoptosis (programmed cell death).[4]

Emissive lanthanide complexes constitute an emerging class of metal-based emissive probes that are being examined actively at present for use in cellulo.[5–8] Typically, these lanthanide complexes incorporate a sensitising chromophore that absorbs light efficiently (high ε) and facilitates emission from the proximate Ln(III) ion, following intramolecular energy transfer. Here, we describe Eu and Tb(III) complexes of two constitutionally isomeric dibasic ligands H_2L^1 and H_2L^2 that possess two *trans*-related azaxanthone chromophores. These chromophores are effective sensitising moieties for lanthanide emission;[9,10] the latter creates a greater steric demand at the metal centre in the derived Ln(III) complexes, $[Ln{\cdot}L^1]^+$ and $[Ln{\cdot}L^2]^+$. Furthermore, we report the surprising behaviour of the monocationic luminescent lanthanide(III) complexes $[Ln{\cdot}L^2]^+$. The Eu and Tb complexes bind to serum albumin changing the coordination environment and remain strongly emissive; the Tb analogues stain the cell nucleus selectively, allowing the monitoring of nuclear DNA changes in dividing cells, as mitosis progresses.

Reaction of the nicotinic acid derivative, **1a**,[11] with $POCl_3$ gave the 4-chloropyridine, **1b** (56%). Treatment with sodium phenoxide followed by cyclisation in polyphosphoric acid afforded the 3-azaxanthone, **2a** (50%). Benzylic bromination (NBS, CCl_4) allowed isolation of **2b**. Ligands H_2L^1 and H_2L^2 were obtained by reaction of **2b** and **3**, respectively, with the bis-^{t}Bu ester of 1,4,7,10-tetraazacyclododecane-1,7-diacetic acid (DO2A), followed by deprotection with TFA (CH_2Cl_2, 20 °C). Complexation with $Ln(OAc)_3$ (pH 6, H_2O, 50 °C), followed by ion-exchange (Dowex Cl^- resin), led to isolation of the monocationic complexes. Measurements of excited state lifetimes in H_2O and D_2O allowed an estimation of the metal hydration state, Table 1. For $[Ln{\cdot}L^1]^+$, both the Eu and Tb centres are coordinated to one water molecule, whereas for $[Ln{\cdot}L^2]^+$, no coordinated waters are present,[13] consistent with the increased steric demand of the bound 1-azaxanthone moiety.



Incremental addition of human serum albumin (HSA) to $[Eu{\cdot}L^1]^+$ led to a 20% reduction in the Eu emission lifetime (0 to 0.5 mM) echoed by a reduction in the observed emission intensity, with no change in spectral form. In contrast, parallel experiments with $[Eu{\cdot}L^2]^+$ revealed a dramatic change in the Eu spectral emission profile, accompanied by a 50% increase in the Eu excited state lifetime (Fig. 1 and ESI†). A similar pattern of behaviour was observed in titrations with $[Tb{\cdot}L^1]^+$ and $[Tb{\cdot}L^2]^+$. In the latter case, the emission lifetime increased by 65% following HSA addition, and in the former the overall lifetime reduced by a factor of 90% and the emission intensity

[a] *Department of Chemistry, Durham University, South Road, Durham, UK DH1 3LE. E-mail: david.parker@dur.ac.uk*
[b] *Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hunghom, Hong Kong, China*

† Electronic supplementary information (ESI) available: Ligand and complex synthesis and emission spectra; spectral titrations of lanthanide complexes with human serum albumin; experimental details of cell culture and microscopy. See DOI: 10.1039/b924679g

Table 1 Radiative rate constants (k/ms^{-1}) and quantum yields for lanthanide complexes (295 K, pH 5.5)[a]

Complex	ϕ_{em}	k_{H_2O}/ms^{-1}	k_{D_2O}/ms^{-1}	q[b]
[Eu·L^2]Cl	18	1.02	0.65	0.14
[Tb·L^2]Cl	36	0.43	0.36	0.05
[Eu·L^1]Cl	10	1.79	0.64	1.1
[Tb·L^1]Cl	34	0.66	0.36	1.2

[a] Measurements were made as reported in ref. 12, with 10% errors on k and 20% errors on ϕ values. [b] Values of q (±20%) are derived using methods in ref. 13.

fell by a factor of two† (zero to 0.7 mM HSA). The key observation in these titrations is the change in spectral form with [Eu·L^2]$^+$, following protein addition. This can only occur if there is a change in the metal coordination environment.[14] The large increase in the $\Delta J = 2$ transitions (610–620 nm, Fig. 1) and the change in the $\Delta J = 1$ manifold splitting are consistent with dissociation of one sensitiser, replacing the neutral N donor by a polarisable charged oxygen donor derived from the protein, *e.g.* an Asp or Glu side chain carboxylate group.[5a,14]

The variation of lanthanide emission intensity and lifetime was also studied in a series of titrations of [Ln·L^n]$^+$ (n = 1,2; Ln = Eu, Tb) with calf thymus DNA (42% CG base pairs), adding the DNA to the complex, up to a ratio of 4 base pairs per complex. No changes (±10%) in emission lifetime and intensity were observed for [Eu·L^1]$^+$ and [Eu·L^2]$^+$. Parallel changes in absorption and excitation spectra were monitored and no significant change occurred in the wavelength or absorbance of the azaxanthone chromophore band at 336 nm. Addition of DNA to each Tb complex also caused neither a spectral shift nor any hypochromism in the azaxanthone chromophore as observed by absorption and excitation spectroscopy, consistent with a binding interaction that is not primarily intercalative in nature. In each case, the emission lifetime and intensity were both reduced by about 50% at 1 base pair per complex. Previous studies examining the behaviour of various lanthanide(III) complexes in the presence of several nucleic acids have revealed charge transfer quenching of lanthanide emission,[12,15] with Tb complexes

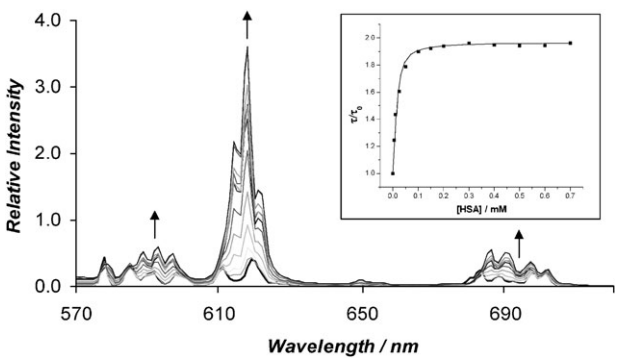


Fig. 1 Variation of europium emission spectral form for [Eu·L^2]Cl (20 μM) with added human serum albumin (λ_{exc} 340 nm, 298 K, pH 7.4, 10 mM HEPES, 10 μM NaCl). The inset shows the concomitant increase in the Eu emission lifetime, showing the fit (line) to the observed data for log K = 5.25 (±0.05); a parallel titration with [Eu·(H$_2$O)L^1]Cl gave log K = 3.55 (±0.03).

much more sensitive to a quenching process that may occur *via* exciplex formation between the chromophore and the electron rich CG base pairs. Consistent with this idea, addition of poly(dAdT) to either [TbL^1(H$_2$O)]$^+$ or [Tb·L^2]$^+$ led to a 20% smaller reduction in the intensity of the lanthanide emission.

The complexes [Tb·L^2]$^+$ and [Tb·L^1]$^+$ were examined in cell uptake experiments using HeLa cells, observing cellular staining by luminescence microscopy. Using relatively high concentrations of added complex in the medium (range 10 to 100 μM), the pattern of staining was consistent with an endosomal/lysosomal distribution. However, at much lower added concentrations of complex (*e.g.* <1 μM [Tb·L^2]$^+$ or [Tb·L^1]$^+$, 5 to 30 min incubation) less than 10% of the observed cells were stained. The pattern of staining and the nature of the nuclear localisation profile (Fig. 2) were consistent with preferential labelling of those cells undergoing division in the 'M' phase, where membrane integrity is somewhat compromised. This hypothesis was supported by co-staining experiments using [Tb·L^2]$^+$ and Hoechst 33342 (each 0.5 μM in medium, 2 h incubation; λ_{exc} 350 nm). The latter stained all cells observed but the Tb complex only stained selected cells, including those in mitosis. Cells at each stage of the mitotic cycle were discerned, *e.g.* after nuclear envelope breakdown at prophase, prometaphase, metaphase and at telophase, with chromosomal DNA stained selectively.[1,2]

A separate experiment at higher resolution attempted to follow the fate of a single cell with time (5 min intervals), following incubation of the complex in the cell growth medium for 3 h (50 nM, [Tb·L^2]$^+$). Changes in the packaging and assembly of chromatin from prometaphase to metaphase were clearly discerned (Fig. 3), although the study was prematurely halted, after 1 h prior to the onset of anaphase. This is most likely due to phototoxicity, associated with the intensity of

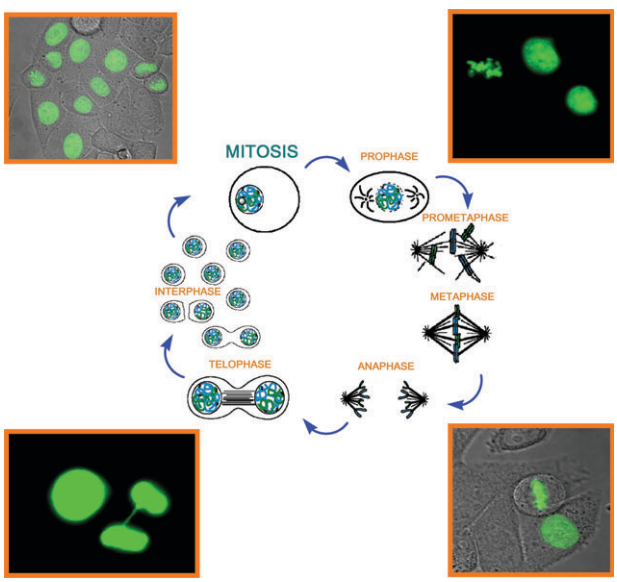


Fig. 2 Bright field (upper left and lower right) and fluorescence microscopy images of HeLa cells undergoing mitosis, with an illustrative schematic of the cell cycle (0.5 μM, [Tb·L^2]Cl, 3 h, λ_{exc} 350 nm).

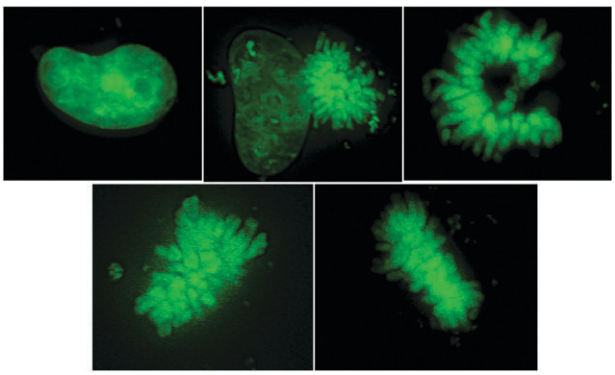


Fig. 3 Time course (5 min intervals) of fluorescence microscopy images staining chromatin in a cell undergoing division, showing the progression from interphase (top left for comparison) *via* prophase to prometaphase and metaphase (50 nM $[Tb{\cdot}L^2]Cl$, scale bar 5 μM, HeLa cell, λ_{exc} 300 nm).

illumination, leading to spindle checkpoint activation and a pause in the observed cell cycle. In control experiments, assessing the cellular toxicity of $[Tb{\cdot}L^1]^+$ and $[Tb{\cdot}L^2]^+$, IC_{50} values of >400 μM were estimated, based on the perturbation of mitochondrial redox (using 'MTT' or 'WST-1' methods). Therefore, future studies with such probes will focus on using less intense irradiation or will use two photon microscopy methods, with excitation wavelengths in the near-IR range of 710 to 780 nm that are less likely to create problems of phototoxicity.

In summary, the low chemical toxicity and ability of the terbium complexes to stain the nuclear DNA in mitotic cells augur well for the development of these and related metal-based optical probes[8b] that are capable of visualising DNA events in living cells. Future work will also address the mechanism of cell uptake at low added complex concentration in various cell types. For the cells examined here, this is likely to be different from the pathway of macropinocytosis[5a] defined for many lanthanide complexes recently.

We thank EPSRC and CISbio s.a. for support.

Notes and references

1 D. Zink, N. Sadoni and E. Stelzer, *Methods*, 2003, **29**, 42; F. Mora-Bermudez and J. Ellenberg, *Methods*, 2007, **41**, 158.
2 T. Haraguchi, T. Kaneda and Y. Hiraoka, *Genes Cells*, 1997, **2**, 369.
3 T. Hirota, D. Gerlich, B. Koch, J. Ellenberg and J.-M. Peters, *J. Cell Sci.*, 2004, **117**, 6435.
4 A. Khodjakov and C. L. Rieder, *Methods*, 2006, **38**, 2.
5 (*a*) C. P. Montgomery, B. S. Murray, E. J. New, R. Pal and D. Parker, *Acc. Chem. Res.*, 2009, **42**, 925; (*b*) E. J. New, D. Parker, D. G. Smith and J. W. Walton, *Curr. Opin. Chem. Biol.*, 2010, DOI: 10.1016/j.cbpa.2009.10.003; (*c*) R. Pal and D. Parker, *Org. Biomol. Chem.*, 2008, **6**, 1020; (*d*) B. S. Murray, E. J. New, R. Pal and D. Parker, *Org. Biomol. Chem.*, 2008, **6**, 2085.
6 A. S. Chauvin, S. Comby, B. Song, C. D. Vandevyver, F. Thomas and J.-C. G. Bunzli, *Chem.–Eur. J*, 2007, **13**, 9515; C. P. Montgomery, E. J. New, L. O. Palsson, D. Parker, A. S. Batsanov and L. Lamarque, *Helv. Chim. Acta*, 2009, **92**, 2186.
7 S. W. Botchway, M. Charnley, J. W. Haycock, A. W. Parker, D. L. Rochester, J. A. Weinstein and J. A. G. Williams, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 16071; P. Wu, E. L. M. Wong, D. L. Ma, G. S. M. Tsang, K. M. Ng and C. M. Che, *Chem.–Eur. J.*, 2009, **15**, 3652; C. A. Puckett and J. K. Barton, *J. Am. Chem. Soc.*, 2009, **131**, 8738; V. Fernandez-Moreira, F. L. Thorp-Greenwood and M. P. Coogan, *Chem. Commun.*, 2010, **46**, 186.
8 (*a*) N. A. O'Connor, N. Stevens, D. Samaroo, M. R. Soloman, A. A. Marti, J. Dyer, H. Vishwasrao, D. L. Atkins, E. R. Kandel and N. J. Turro, *Chem. Commun.*, 2009, 2640; A. J. Amoroso, R. J. Arthur, M. P. Coogan, J. B. Court, V. Fernandez-Moreira, A. J. Hayes, D. Lloyd, C. Millet and S. J. A. Pope, *New J. Chem.*, 2008, **32**, 1097; (*b*) For ruthenium-based nuclear DNA staining: M. R. Gill, J. Garcia-Lara, S. J. Foster, C. Smythe, G. Battaglia and J. A. Thomas, *Nat. Chem.*, 2009, **1**, 662.
9 P. Atkinson, K. S. Findlay, F. Kielar, R. Pal, D. Parker, R. A. Poole, H. Puschmann, S. L. Richardson, P. A. Stenson, A. L. Thompson and J. Yu, *Org. Biomol. Chem.*, 2006, **4**, 1707.
10 A. Thibon and V. C. Pierre, *J. Am. Chem. Soc.*, 2009, **131**, 434.
11 E. E. Kilbourn and M. C. Siedel, *J. Org. Chem.*, 1972, **37**, 1145.
12 G.-L. Law, D. Parker, S. L. Richardson and K.-L. Wong, *Dalton Trans.*, 2009, 8481.
13 A. Beeby, I. M. Clarkson, R. S. Dickins, S. Faulkner, D. Parker, L. Royle, A. S. de Sousa, J. A. G. Williams and M. Woods, *J. Chem. Soc., Perkin Trans. 2*, 1999, 493.
14 R. S. Dickins, J. I. Bruce, D. Parker and D. J. Tozer, *Dalton Trans.*, 2003, 1264; D. Parker, *Chem. Soc. Rev.*, 2004, **33**, 156.
15 G. Bobba, J.-C. Frias, M. J. Cann, D. Parker and C. J. Hutchison, *Org. Biomol. Chem.*, 2003, **1**, 905; R. A. Poole, G. Bobba, M. J. Cann, J.-C. Frias, D. Parker and R. D. Peacock, *Org. Biomol. Chem.*, 2005, **3**, 1013.