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Luminescent nonacoordinate cationic lanthanide complexes as potential cellular imaging and reactive probes

Juan C. Frias,[a] Gabriella Bobba,[a] Martin J. Cann,[b] Chris J. Hutchison[b] and David Parker*[a]

[a] *Department of Chemistry, University of Durham, South Road, Durham, UK DH1 3LE. E-mail: David.Parker@durham.ac.uk*

[b] *School of Biological Sciences, University of Durham, Science Laboratories, Durham, UK DH1 3LE*

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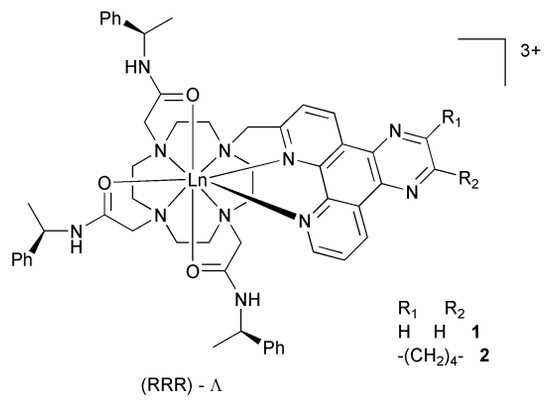
Nonacoordinate Δ- and Λ-Eu and Tb complexes have been tested as imaging and reactive probes in mouse fibroblast (NIH 3T3) cells. The uptake of these complexes by the cells was assessed by fluorescence microscopy. Complex-induced DNA damage was studied by gel electrophoresis and shown to be a function of complex chirality.

Lanthanide coordination complexes have found application in such diverse methods of analysis as luminescence spectroscopy, NMR and EPR due to their unique magnetic and optical properties. Luminescence based methods are particularly suited to the study of biochemical systems, because of the variety of possible applications and the relatively simple instrumentation required.[1] Examples include the development of time-resolved immunoassays[2] and the use of lanthanide systems as long-lived donors in fluorescence resonance energy transfer (FRET) analyses.[3] Recently, significant advances in the spectral analysis of tissue have been developed.[4] Fluorescence imaging coupled to conventional endoscopy offers advantages for diagnosis.[5] Although improvements are still necessary, in order to reach the goal of clinical practicality, these investigations demonstrate the merit of performing spectral analysis *in vivo*. Image contrast agents for use *in vivo* should exhibit the following properties: fluorescence at wavelengths shifted from the excitation wavelength, preferential uptake by different cell types or cell compartments, no toxicity, and high chemical stability with respect to complex degradation.[6]

Here, we report the properties of some enantiopure nonacoordinate cationic lanthanide complexes *in vitro* (**1**, dpq, and **2**, dpqC) and their interactions with a mouse fibroblast cell line. The tetraazatriphenylene chromophore attached to these complexes not only acts as an efficient sensitiser for Eu^{3+} and Tb^{3+} emission but also may intercalate between the base pairs of the DNA. The complexes have an absorbance maximum at 340 and 350 nm and emit with a millisecond lifetime in the range 480 to 720 nm. Such features avoid problems associated with autofluorescence and Rayleigh scattering. Each complex also possesses a high overall quantum yield (ϕ_{Eu} = 0.16, ϕ_{Tb} = 0.40 in water, 295 K).[7]



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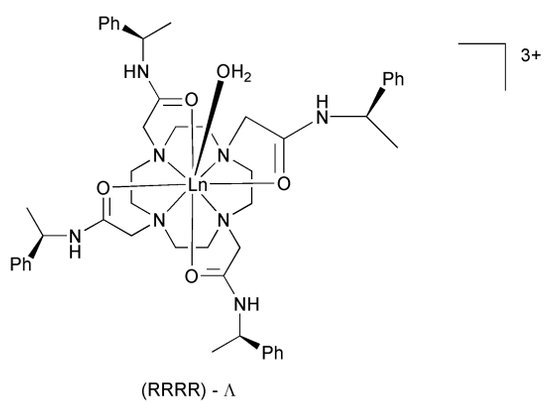

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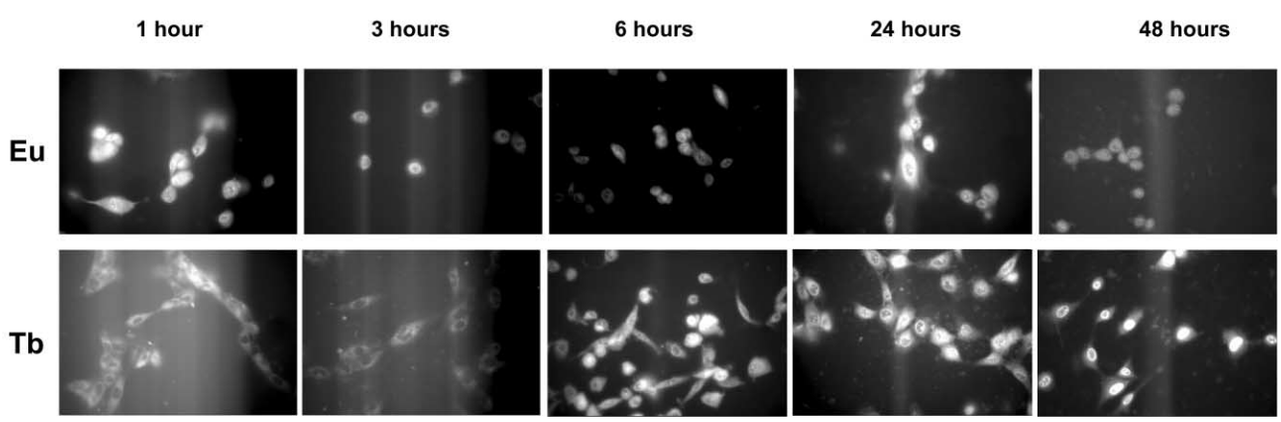

Fig. 1 Microscopy images for the internalization of the Eu and Tb probes at different times.

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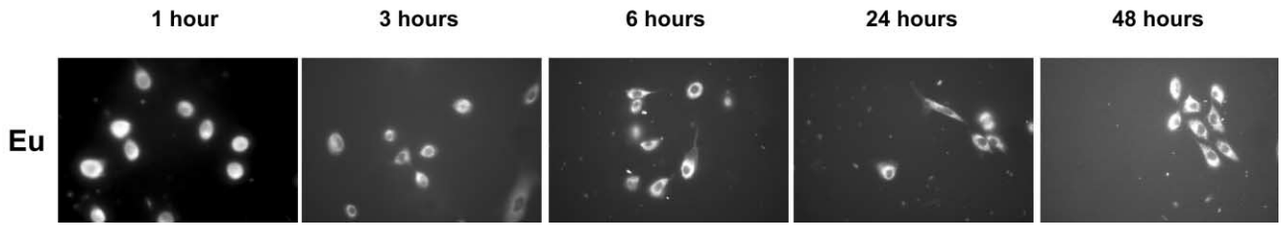

Fig. 2 Microscopy images for the transit of Eu probe after 3 hours incubation and washing of unbound complex.

NIH 3T3 cells were cultured (Dulbecco's Modified Eagle Medium supplemented with 10% new-born calf serum, 50 μg mL^{-1} penicillin and streptomycin, and non-essential amino acids) on 10 mm diameter cover slips. The cells were incubated with an aqueous solution of the complex (1 mM). Cells were examined by fluorescence microscopy from 1 to 48 hours after complex loading.[8] A time-course collection of images showed that cells internalise each complex but that uptake of the Eu complex appears faster than the Tb analogue. The microscopy images (Fig. 1) reveal the migration of the probes through the cytoplasm, across the nuclear membrane, and into the nucleus, showing substructures within the nucleus. Surprisingly, the Tb complex appeared to remain in the cytoplasm for longer periods than the Eu and was apparently slower to migrate to the nucleus. However, earlier *in vitro* studies had indicated that emission from the Tb complexes was quenched to a greater extent when bound to DNA than for the Eu analogues. The luminescence observed in the different images corresponds to the emission from the excited state of the lanthanide ion. The known efficiency of inter-system crossing with this sensitising chromophore, high quantum yields that the complexes possess, and the utilization of different cut-off filters ruled out any fluorescence associated with emission from the heterocyclic chromophore. When uptake of the Δ and Λ enantiomeric complexes was compared, no significant differences were discerned.

In a separate experiment, the cells were inoculated for a period of 3 hours, to ensure that the complex had been internalised and was located in the nucleus (Fig. 2). After inoculation, unbound complex was removed by washing with phosphate-buffered saline solution. The images show a transfer of the luminescence from the nucleus to the cytoplasm after a few hours and indicate that the complex remains in the cytoplasm for sustained periods. The egress of the complex in the presence of excess complex from the nucleus back into the cytoplasm was not observed earlier (Fig. 1). Such behaviour suggests that entry into the cell nucleus requires a favourable concentration gradient of the complex.

The mechanism by which the cells take up the complex has yet to be defined. These probes (tripositive charge and MW > 1000) are unlikely to enter by any passive diffusion process across the plasma membrane and therefore active uptake is more likely. No vesicular structures were observed in the cytoplasm by fluorescence microscopy, so an endocytotic uptake mechanism seems unlikely. The mechanism of uptake may involve a transporter or pore in the membrane.

The observation of luminescence in the cell nucleus suggests that these lanthanide complexes may be useful structural or reactive probes and that irradiation of the complex at 340 or 350 nm, when located in the nucleus may lead to DNA damage and apoptosis. Irradiation of cultured cells pre-incubated with the europium probe for 45 minutes (350 nm) resulted in 100% cell death as assessed by Trypan Blue exclusion assay after an incubation of 18 hours. In a control experiment, less than 15% cell death was observed over the same period in the absence of direct irradiation or in the absence of complex. Irradiation of a pBlueScript based plasmid DNA in the presence of the Eu or Tb complex (1 mg mL^{-1}) for different periods of time was monitored by gel electrophoresis (0.8% agarose). Separation of the different DNA species by gel

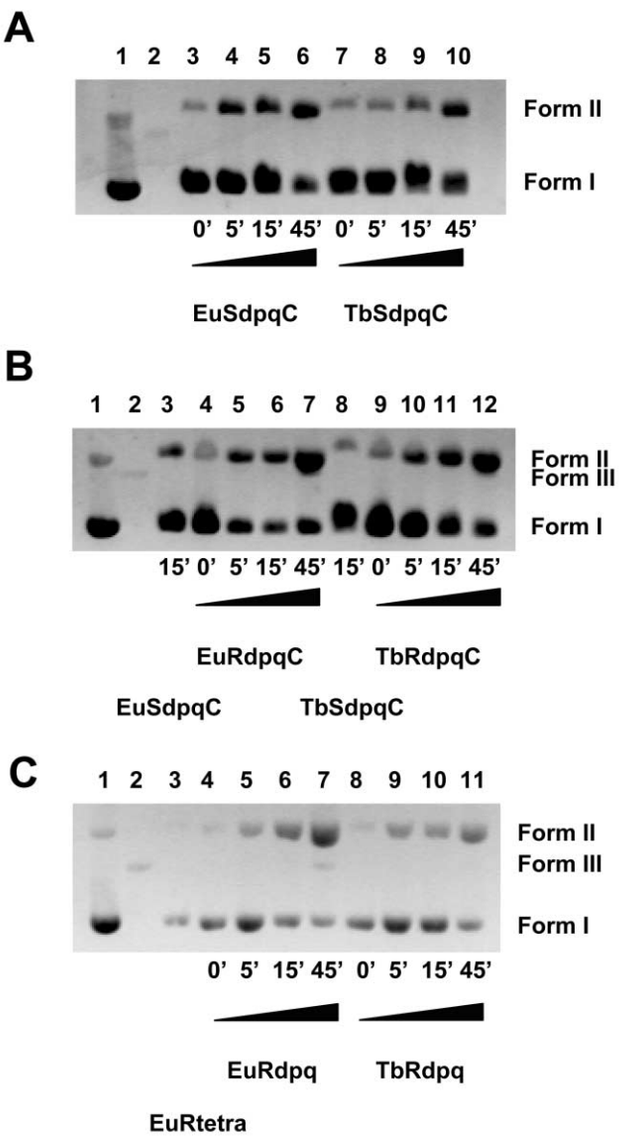

Fig. 3 Agarose gel electrophoresis of pBlueScript plasmid treated with various complexes: **A** 1 mg mL^{-1} EuSdpqC, and TbSdpqC. Lane 1 is the supercoiled pBlueScript plasmid; lane 2 is the linear plasmid; lanes 3–6 correspond to the plasmid in presence of EuSdpqC irradiated at 350 nm for 0, 5, 15, and 45 minutes; lanes 7–10 represent plasmid in presence of TbSdpqC irradiated at 350 nm for 0, 5, 15, and 45 minutes. **B** 1 mg mL^{-1} EuRdpqC and TbRdpqC. Lane 1 is the supercoiled pBlueScript plasmid; lane 2 is the linear plasmid; lane 3 is a control consisting of EuSdpqC and plasmid irradiated for 15 minutes; lanes 4–7 correspond to the plasmid in presence of EuRdpqC irradiated at 350 nm for 0, 5, 15, and 45 minutes; lane 8 is a control experiment consisting of plasmid and TbSdpqC irradiated for 15 minutes; lanes 9–12 correspond to plasmid in presence of TbRdpqC irradiated at 350 nm for 0, 5, 15, and 45 minutes. **C** 1 mg mL^{-1} EuRdpq and TbRdpq. Lane 1 is the supercoiled pBlueScript plasmid; lane 2 is the linear plasmid; lane 3 is a control consisting of Λ-Eu tetraamide, **3**, and plasmid irradiated for 15 minutes; lanes 4–7 correspond to the plasmid in presence of EuRdpq irradiated at 340 nm for 0, 5, 15, and 45 minutes; lanes 8–11 correspond to the plasmid in presence of TbRdpq irradiated at 340 nm for 0, 5, 15, and 45 minutes.

electrophoresis revealed evidence for selective DNA damage, indicated by the formation of nicked DNA (form II) and then linear DNA (form III), Fig. 3. It can be seen that with increasing irradiation time, the extent of formation of nicked DNA increases gradually, while supercoiled DNA (form I) decreases (Fig. 3). This effect is most clearly observed for the Λ-complexes: in particular, only the Λ-complexes gave rise to formation of linear DNA (form III), after 45 minutes irradiation. In control experiments, irradiation for 45 minutes at 350 nm in the absence of complex gave rise to no observable change. Moreover, in the presence of the chiral tetraphenylamide complex, **3**, neither nicked nor linear DNA was observed following irradiation.

The mechanism of this nicking activity is unknown. Rate constants for depopulation of the lanthanide excited state in the absence/presence of DNA had earlier revealed the absence of any bound water molecule[7] that could be involved in phosphate bond cleavage *via* a hydrolytic mechanism.[9] Furthermore, no evidence for singlet oxygen formation was found in the absence or presence of DNA. Given that the lanthanide excited state itself is deactivated on binding to DNA, as revealed by a reduction in the lifetime of the excited state (Tb > Eu, Λ > Δ),[7] it is tempting to speculate that the observed damage to DNA may be associated with a mechanism based on a Ln^{4+} transient. This may be produced in the excited state as a consequence of a metal-to-ligand charge transfer interaction involving the tetraazatriphenylene chromophore that is sufficient to oxidise guanine or GG diads in the DNA.[10] Further time-resolved studies are required to address this possibility.

In summary, a new family of strongly luminescent probes has been defined based on cationic nonacoordinate lanthanide complexes. Europium and terbium complexes are quickly taken up by NIH 3T3 cells and localise in the cell nucleus and around the cell membrane, after a few hours. A transit from the cytoplasm to the nucleus was observed and some substructure features within the nucleus could also be defined. Plasmid supercoiled DNA is damaged on photolysis at 340 and 350 nm producing nicked (form II) and linear (form III) DNA as a function of time; the extent and nature of damage are also a function of the chirality of the complex. As DNA damage is known to induce apoptotic cell death, such behaviour suggests that the cell death observed on photoirradiation may be a consequence of programmed cell death rather than by a non-specific necrotic mechanism. Such complexes may therefore be considered for development as therapeutic probes, for example in the treatment of accessible tumours, such as skin melanoma.

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