Two-photon microscopy study of the intracellular compartmentalisation of emissive terbium complexes and their oligo-arginine and oligo-guanidinium conjugates†

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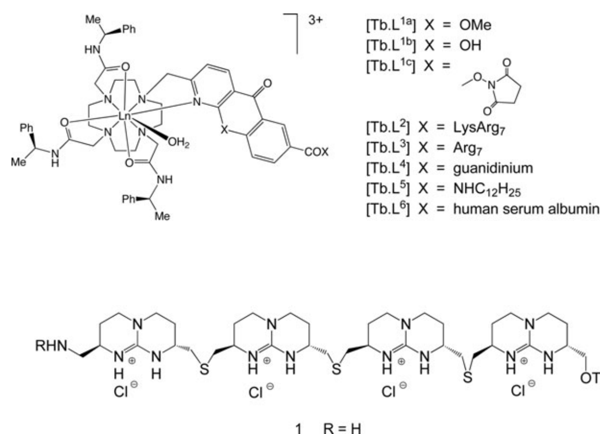
An emissive terbium complex has been conjugated to a C_{12} chain, Lys-Arg_7, Arg_7, a tetraguanidinium cation and human serum albumin; two-photon excitation at 720 nm facilitated microscopy studies revealing cell localisation profiles with the oligo-guanidinium conjugate localising in mitochondria but causing apoptotic cell death (IC_{50}12 μM), the C_{12}-amide complex giving rise to necrotic cell death in skin fibroblasts (IC_{50} 8 μM) and the peptide conjugates and the methyl ester generating punctuate cytosolic intracellular distributions.

A key issue in the development of optical imaging probes of the cellular environment is the ability to transport an imaging probe into the cell and direct it to a particular cellular compartment. There are two main strategies by which this problem can be addressed. The first requires the conjugation of a targeting vector that will facilitate cell entry and may promote selective localisation in a given organelle. Alternatively, the chemical structure of the emissive probe can be systematically varied and the localisation profile screened empirically in a variety of cell types. Examples of the former approach are largely based on arginine-rich, cell-penetrating peptides and analogues thereof,[1–3] and have been extended to various oligo-guanidinium systems, that are more resistant to peptidase or enzymatic metabolism and exhibit a marked tendency to localise in mitochondria, notwithstanding significant toxicity.[4] The second approach characterises the development of many of the fluorescent intracellular stains that are commonly used in cell biology to visualise particular cell compartments.[5]

Over the past few years, long-lived luminescent probes of the Ln(III) ions have been devised, and more recently shown to localise within living cells by fluorescence microscopy.[6–9] Targeted terbium complexes have been explored, in which an integral α-chlorophenylisoquinoline moiety was linked to the ligand structure in an attempt to favour receptor binding at the outer mitochondrial membrane.[6] Alternatively, it has become apparent that the sensitising moiety itself, required for long wavelength excitation of the coordinated Ln(III) ion, may serve to direct the uptake profile and intracellular localisation of the probe.[7a,8] The polycyclic heteroaromatic sensitiser determines protein affinity and hence can markedly influence uptake and intracellular transport. Examples have been reported in which the probe is directed to the nucleoli/ribosomes,[7b,8] the nuclear chromosomal DNA[7a] and the perinuclear endosomal and lysosomal sites.[7c,9]

In this communication, we report the outcome of preliminary studies evaluating the cellular localisation profile of five different conjugates linked to an emissive terbium complex. The starting point for this work was the terbium complex, $[Tb.L^{1a}]Cl_3$, ($\phi_{em}(H_2O)$ = 20%), which incorporates an azaxanthone chromophore as the sensitising moiety[10] and possesses three relatively bulky and lipophilic α-phenylethylamide arms, which have been shown to suppress intermolecular quenching either by low MW reductants (*e.g.* ascorbate or urate) or by proteins.[11,12] Microscopy studies have been undertaken using both one- and two-photon excitation (λ_{exc} 720 nm), exemplifying this approach for the first time with terbium systems.[13]



The terbium complex, $[Tb.L^{1a}]Cl_3$, was prepared as described recently.[10,12] Controlled base hydrolysis at pH 10 (H_2O/20 °C/24 h) afforded the acid, $[Tb.L^{1b}]Cl_3$, which was converted into the *N*-hydroxysuccinimide ester $[Tb.L^{1c}]Cl_3$ by

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† Electronic supplementary information (ESI) available: Experimental section giving synthesis, characterisation, cell culture, toxicity and microscopy details. Fig. S1: Plot of absorbance *vs.* emission intensity for selected complexes. See DOI: 10.1039/b803864c

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EDC coupling (DMSO, 60%) in 100 mg batches.[11] The active ester was added to the peptides, Lys-Arg$_7$ and Arg$_7$ and to human serum albumin (HSA) in aqueous HEPES buffer (0.1 M, pH 7.4, 24 h) to afford the desired conjugates, [Tb.L^2], [Tb.L^3], [Tb.L^6]. The former two were purified by preparative reverse-phase HPLC and the latter by dialysis. Similarly, reaction of [Tb.L^{1c}] in dry DMF with the lipophilic tetraguanidinium cation, **1**, (as its chloride salt) or with dodecylamine, afforded [Tb.L^4] and [Tb.L^5], respectively, following precipitation onto dry ether and HPLC purification. In each case, the lifetime of the terbium emission was of the order of 1.6 ms, consistent with an absence of significant dynamic quenching of the lanthanide excited state.[11,12] However, the overall emission quantum yields differ by an order of magnitude (pH 7.4, 298 K) and were in the order: [Tb.L^{1a}] (ϕ_{em} = 20%) > [Tb.L^5] (9%) > [Tb.L^4] ~ [Tb.L^3] ~ [Tb.L^2] (2%). This was borne out by plotting the overall terbium emission intensity as a function of absorbance at 340 nm (range 0.02–0.15); the gradients of these plots echoed the measured ϕ_{em} variation. Evidently, there must be a static quenching mechanism, lowering the overall quantum yield. This may tentatively be attributed to an intramolecular charge transfer process, quenching the excited state of the azaxanthone chromophore.

The cell uptake and localisation profiles were examined in live Chinese hamster ovarian cells (CHO), mouse skin fibroblasts (NIH-3T3) and carcinoma (HeLa) cells using fluorescence microscopy, following single- or two-photon excitation of the azaxanthone chromophore. Using appropriate filter sets, terbium emission was readily detected and each complex exhibited <3% azaxanthone fluorescence, consistent with earlier observations.[11,12] Incubation of the oligo-arginine terbium complexes (20 μM [Tb.L^2] or [Tb.L^3]) in both CHO and NIH-3T3 cells led to rapid uptake of the complex and microscopy images taken within 10 min of loading revealed a punctuate distribution, that by 4 h of incubation was consistent with localisation in the perinuclear endosomes and lysosomes. The methyl ester complex, [Tb.L^{1a}],[3] was also rapidly

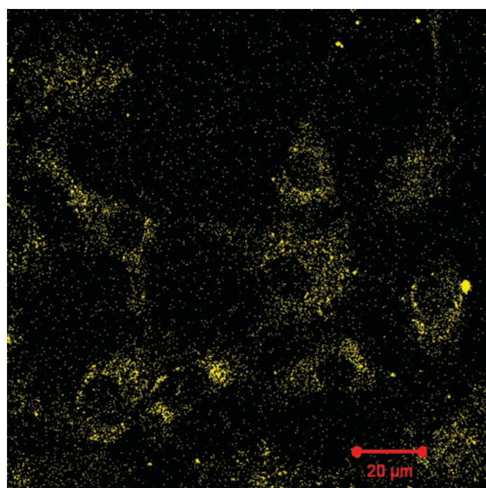

Fig. 1 Two-photon microscopy image of NIH-3T3 cells stained with [Tb.L^{1a}]Cl$_3$, recorded after 5 h (20 μM complex), showing the distribution of the complex throughout the cytosol (scale bar 20 microns).

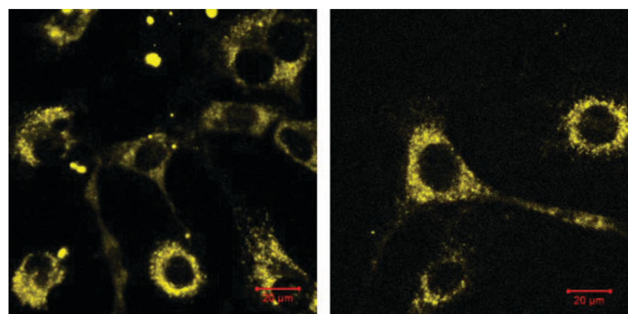

Fig. 2 Two-photon fluorescence microscopy images (λ_{exc} = 720 nm) of NIH-3T3 cells stained with [Tb.L^3] (left) and [Tb.L^4] (right), recorded 3 h after loading (10 μM complex; scale bar 20 microns).

internalised by NIH-3T3 cells and was quite widely distributed throughout the cytosol at a 5 h time point, as revealed by two-photon microscopy, following excitation at 720 nm (Fig. 1). At later time points (12, 24 h), a greater degree of compartmentalisation was noted, consistent with an increasingly endosomal/lysosomal profile in the perinuclear region, confirmed by co-localisation experiments with Lysotracker[5] dyes. A comparative study of the uptake of [Tb.L^{1a}][3] and [Tb.L^3] in HeLa cells was also undertaken, using two-photon microscopy. After a one hour incubation period (10 μM complex), the methyl ester complex was readily observed, giving images that were approximately three or four times more intense than those for [Tb.L^3] (Fig. 2). In each case, the localisation of the probe is mainly in the perinuclear region.

Experiments with the oligo-guanidinium conjugate, [Tb.L^4], and the dodecylamide complex, [Tb.L^5], were compromised by the toxicity of the probe. Measurements of the IC$_{50}$ values were made using an MTT assay over a 24 h cell incubation period,[14] giving values of 12 and 8 μM, respectively. These compared to the IC$_{50}$ values of 78 μM for [Tb.L^{1a}] and 148 μM for [Tb.L^{1b}]. Thus, microscopy experiments with [Tb.L^5] in NIH-3T3 cells were inhibited by the observation of significant cell death. However, this complex was much better tolerated by CHO cells, and localisation to the Golgi apparatus and endoplasmic reticulum was suggested. The oligo-guanidinium conjugate exhibited rapid internalisation and in a two-photon microscopy experiment gave a profile after 3 h that was consistent with the mitochondrial distribution observed earlier with its fluorescein conjugate.[4] More intense staining was

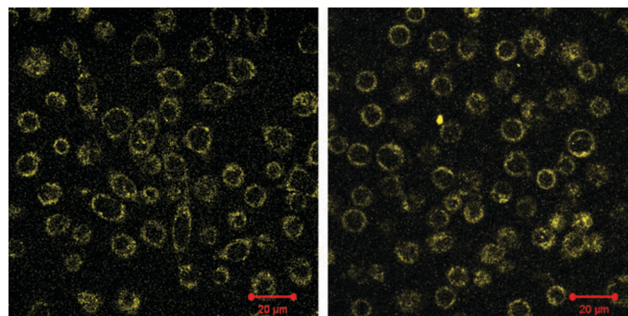

Fig. 3 Two-photon microscopy images (1 h post incubation, λ_{exc} = 720 nm) of HeLa cells stained with [Tb.L^{1a}]Cl$_3$ (left), and [Tb.L^3] (right) showing the localisation of the terbium emission from the complex (scale bar 20 microns).

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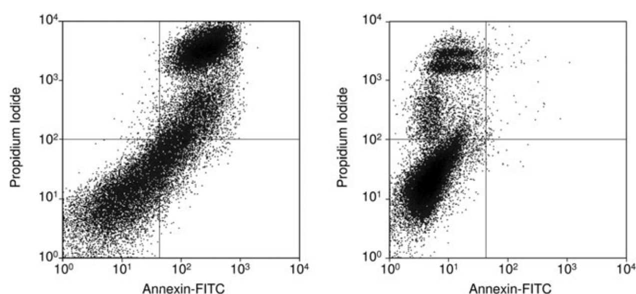

Fig. 4 Left: flow cytometry plot of NIH-3T3 cells, 4 h after incubation with the oligo-guanidinium conjugate, [Tb.L^4]; right: plot of CHO cells after 24 h incubation with the dodecylamide, [Tb.L^5]$^{3+}$, indicating necrotic cell death. (In each case flow cytometry 'dot images' correlate fluorescence of an Annexin-FITC conjugate (apoptosis marker) on the *x*-axis, with propidium iodide fluorescence (necrosis marker) on the *y*-axis; apoptosis is then revealed in the north east quadrant and necrosis in the north west).

observed after a 12 h incubation period revealing an increasingly endosomal contribution (Fig. 3). With the serum albumin conjugate, [Tb.L^6], no cell uptake was observed, notwithstanding prolonged incubation periods.

The toxicity of [Tb.L^4] and [Tb.L^5] was assessed further by flow cytometry, allowing the distinction between apoptosis (programmed cell death) and necrosis to be assessed.[15] Each complex was studied in CHO and NIH-3T3 cells and results were compared to an untreated control and to controls for necrosis (0 °C, 70% EtOH) and apoptosis (1 μM staurosporine). The complex [Tb.L^4] clearly exhibited apoptosis in each cell line (Fig. 4, left), whilst [Tb.L^5] was not cytotoxic to CHO cells, but in NIH-3T3 cells gave a 'dot plot' at 4 h, (Fig. 4, right), that was consistent with necrotic cell death. Such behaviour may be related to the tendency of the dodecylamide chain to embed itself into membranes and hence destabilise them.

These studies highlight the opportunities available and the problems that may be associated with the development of emissive terbium probes of the cellular environment. The exemplification of two-photon microscopy with terbium probes and the observation of a cellular compartmentalisation profile that can be determined by the nature of the vector used, augurs well for the development of targeted, responsive optical probes. These are preferably based on analogous europium complexes, as the sensitivity of the Eu emission profile to the Eu coordination environment gives rise to changes in the spectral form and circular polarisation of emission, allowing ratiometric analyses.[7,8] Thus, they can be used as responsive probes to signal within living cells, local changes of pH, pX or pM, in real time.

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