Luminescent lanthanide bimetallic triple-stranded helicates as potential cellular imaging probes

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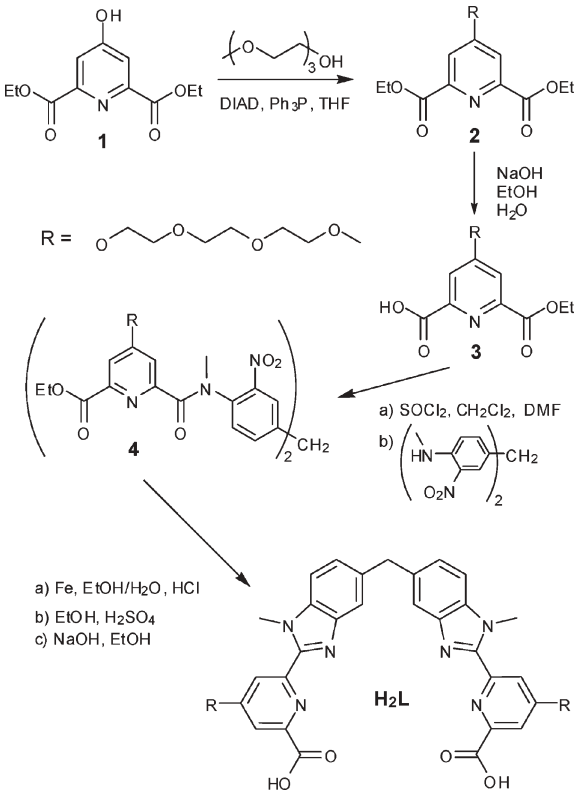
Water-soluble triple-stranded $[Ln_2(L)_3]$ helicates have been successfully tested as imaging probes in human cervical adenocarcinoma cells (HeLa), the complex being not toxic and clearly staining their cytoplasm in a concentration-dependent manner.

Lanthanide ions have provided a new incentive in the development of luminescent molecular probes because of their peculiar properties including easily recognizable line-like emission spectra, long excited-state lifetimes allowing the use of time-resolved measurements, and large Stokes shifts upon ligand excitation.[1] They are now commonly used as luminescent responsive probes in immunoassays[2] and for the quantitative determination of analytes present in biological fluids.[3] Building on these applications, the next step is to integrate them into luminescent stains for bio-imaging purposes.[4] To date, however, only a few labels are available, presumably in view of the numerous requirements to be fulfilled by lanthanide probes for time-resolved applications *in vitro* and *in vivo*. The latter include solubility in aqueous media or in solvents suitable for their conjugation to bio-specific probes, thermodynamic stability, kinetic inertness, and marked sensitization of the metal-centered luminescence. Recently, significant advances in the spectral analysis of tissues have been reported.[5–9] Despite many improvements still being needed to reach clinical practicality, these investigations bring a proof of concept for the use of lanthanide ions in *in vivo* and *in vitro* analyses.

In recent years, we have been tailoring homo- and hetero-bimetallic helicates with the final purpose of designing bi-functional lanthanide probes.[10,11] Although not yet taken advantage of, the intrinsic chirality of the helicates will be an asset in future developments of these probes. Herein, we report the properties of the water-soluble and thermodynamically stable triple-stranded $[Eu_2(L)_3]$ helicate *in vitro*, its interaction with the human cervical adenocarcinoma cell line HeLa and its ability for the imaging of these cells.

The molecular design of the ligand relies on the ditopic hexadentate receptor H_2L (see Scheme 1). To improve the solubility of the neutral bimetallic helicates, polyoxyethylene arms acting as water-solubilizing groups were grafted in the *para* position of the pyridine ring. The substituent in **2** was introduced *via* a Mitsunobu reaction starting from the ethyl diester of dipicolinic acid **1**, which was further converted into a monoester product **3**, followed by Phillips coupling to give **4**. The next step



Scheme 1 Synthesis of the water-soluble ligand.

was a cyclization in presence of iron to form the benzimidazole units. The ester functions were finally hydrolysed to provide the water-soluble H_2L ligand.[12] This receptor exists in two main forms in equilibrium at physiological pH, H_2L and HL^-, as determined by spectrophotometric titration.

The bitopic ligand H_2L self-assembles under stoichiometric conditions with lanthanide perchlorate to yield 2 : 3 Ln : L edifices as the major species at pH 7.4 (in Tris-HCl buffer), as ascertained by spectrophotometric titration and mass spectrometry.[13] The Ln helicates appear to be thermodynamically quite stable with conditional stability constants $\log\beta_{23}$ in the range 26–30 (Ln = La, Eu, Lu).

Upon ligand excitation at 325 nm, the emission spectrum of the neutral $[Eu_2(L)_3]$ helicate displays the usual metal-centered luminescence (Fig. 1, bottom). The energy of the $^3\pi\pi^*$ 0-phonon transition is around 22 100 cm^{-1}, leading to efficient sensitization of the Eu^{III} luminescence, with an overall quantum yield $Q = 18 \pm 2\%$ under physiological conditions, as determined in two different ways, using a comparison method with $Cs_3[Eu(dpa)_3]$ as

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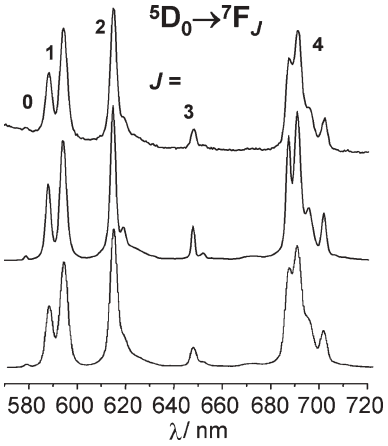

Fig. 1 Corrected emission spectra of $[Eu_2(L)_3]$ in HeLa cells loaded with the helicate 500 μM (after washing with PBS buffer, top), in RPMI-1640 (middle), and 15 μM in water at pH 7.4 (Tris-HCl, bottom); λ_{ex} = 325 nm.

standard[14] and an absolute method with an integration sphere.[15] The Eu($^5D_0$) lifetime is long (τ = 2.4 ± 0.1 ms) and reflects the absence of water molecules in the inner coordination sphere. Working in time-resolved mode avoids problems associated with auto-fluorescence and Rayleigh scattering so that these photophysical properties, long lifetime and sizeable quantum yield, will allow sensitive detection in biological medium. A more detailed analysis of the ligand-field splitting reflected in the transitions to the various ^7F_J levels clearly points to the overall coordination arrangement around the metal ions being derived from D_3 symmetry. In particular, the crystal field splitting of the $^7F_1$ level corresponds to an A_2 (273 cm^{-1}) + split E (453, 479 cm^{-1}) scheme; the latter 26 cm^{-1} splitting is consistent with a relatively weak deviation from the ternary symmetry.

The human cervical adenocarcinoma cell line HeLa was cultured in RPMI-1640 medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino-acids, and 1% 2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid monosodium salt (HEPES buffer).[16]

HeLa cells at 90% confluency were loaded with the helicate (500 μM in RPMI-1640 culture medium) for 6 h, washed with PBS (phosphate buffer saline) ten times, and then harvested with trypsin. The mixture was centrifuged (3000 tr min^{-1}, 5 min), and the pellet was re-suspended in PBS (0.8 mL). The emission spectrum of the Eu^{III} helicate localized in cells displays an overall shape very similar to the emission spectrum in aqueous solution at pH 7.4 (Fig. 1, top). In particular, the intensity ratios of the various transitions are the same (*e.g.* $I(^7F_2)/I(^7F_1)$ = 0.98 as compared to 1.04), and the lifetime remains long (1.7 ± 0.3 ms), thus indicating that the Eu^{III} helicate is un-dissociated inside the cells. The only noticeable difference is an increase in the splitting of the E($^7F_1$) sub-level to 42 cm^{-1}, probably reflecting some distortion of the helical structure due to second-sphere interactions.[17]

The cells grown on glass bottomed cell culture dishes were incubated in cell culture medium with the complex at different concentrations. Cells were examined with a confocal microscope 24 h after complex loading, under 405-nm excitation (argon laser); this was the shortest wavelength available and excitation of the incorporated helicate at this wavelength is made possible because the absorption spectrum extends up to this spectral range and a relatively highly luminous laser source is used. A solution of the helicate in water at pH 7.4 gives exactly the same emission spectrum whatever the excitation wavelength is, 325 or 405 nm.

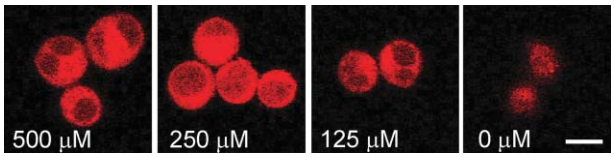

Fig. 2 Cells were incubated in presence of different concentrations of the helicate in RPMI-1640 for 24 h. The images were taken using a Zeiss LSM 500 META confocal microscope (Objective: Plan-Apochromat, 63/1.30 oil; λ_{exc} = 405 nm (Ar laser), LP 505 filter). Scale bar: 17 μm.

The complex $[Eu_2(L)_3]$ clearly permeates into HeLa cells and stains their cytoplasm in a concentration dependent manner (Fig. 2); the higher the concentration, the brighter the images. However, the apparent limiting concentration to get the brightest images is 250 μM; at higher concentration no increase in the image brightness is observed. In addition, some diffuse auto-fluorescence of the cells was observed at 0 μM which in principle could be eliminated by time-gating microscopy (not presently available in our laboratories).

A key question is whether the cells remain viable and healthy over the period of examination. Therefore the influence of the Eu^{III} helicate on cell proliferation and viability was examined by the WST-1 assay[18,19] at time intervals in the range of 30 min to 24 h. As shown in Fig. 3, no significant difference can be observed between the proliferation of the cells in absence or presence of the helicate up to 500 μM. This observation was confirmed by the viability of the cells, after 24 h of incubation with $[Eu_2(L)_3]$: 100% for 0 μM, 89 ± 3% for 125 μM, 101 ± 1% for 250 μM and 108 ± 2% for 500 μM.

Although live cell imaging is a rapidly emerging field, fixed cells are still often used in diagnostic tests. The usefulness of the Eu^{III} helicate for visualizing them is demonstrated in Fig. 4. The cells were fixed with glutaraldehyde (0.4%, 5 min) or using ice-cold methanol (−20 °C, 15 min), resulting in a much higher luminescence than found in living cells.

When the complex is loaded at 4 °C in living cells, we clearly observe its presence in the cytoplasm with luminosity close to that

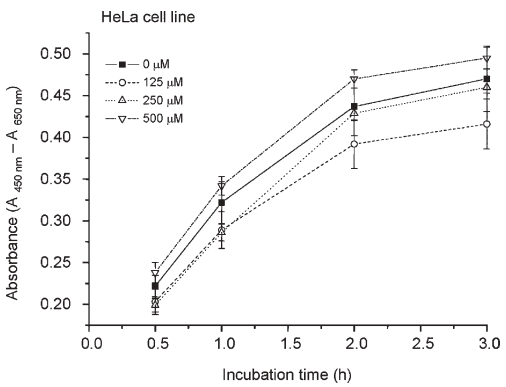

Fig. 3 WST-1 proliferation test of HeLa cells in absence or presence of different concentrations of $[Eu_2(L)_3]$. Each point represents the average over three nominally identical measurements.

Fig. 4 The cells were incubated in presence of 500 μM of the helicate in RPMI-1640 for 24 h. The images were taken as for Fig. 2. Scale bar: 17 μm.

measured at 37 °C. These observations indicate that the helicate is unlikely to enter the cells by non-caveolae and non-clathrin endocytosis and therefore an active uptake is more probable. However, the exact mechanism by which the cells take up the chelate has yet to be unraveled.

Similar experiments are being conducted with the Tb^{III} ($Q = 11.4 \pm 0.3\%$, $\tau = 0.65 \pm 0.05$ ms) and Sm^{III} ($Q = 0.38 \pm 0.06\%$, $\tau = 30.4 \pm 0.4$ μs) helicates and we are also testing the influence of grafting the polyether arm on the benzimidazole unit. In summary, a new family of strongly luminescent and highly stable probes has been developed. The Eu^{III} helicate is taken up by HeLa cells and is localized in the cytoplasm. The luminescent stain can be used in both live and fixed cells imaging with excitation wavelengths up to 405 nm despite its main absorption peaking at 325 nm. Work is in progress to fully study the interaction process with cells, to find better experimental conditions for the imaging, and to take advantage of the long Eu^{III} luminescence lifetime in time-resolved microscopy.

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