Synthesis and cell localization of self-assembled dinuclear lanthanide bioprobes
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Lanthanide bioprobes and bioconjugates are ideal luminescent stains in view of their low propensity to photobleaching, sharp emission lines and long excited state lifetimes permitting time-resolved detection for enhanced sensitivity. In this paper, we expand our previous work which demonstrated that self-assembled dinuclear triple-stranded helicates \([\text{Ln}_2(\text{L}^3\text{C}^2\text{X})_3]\) behave as excellent cell and tissue labels in immunocytochemical and immunohistochemical assays. The synthetic strategy of the hexadentate ditopic ligands incorporating dipicolinic acid, benzimidazole units and polyoxyethylene pendants is revisited in order to provide a more straightforward route and to give access to further functionalization of the polyoxyethylene arms by incorporating a terminal function X. Formation of the helicates \([\text{Ln}_2(\text{L}^3\text{C}^2\text{X})_3]\) \((X = \text{COOH, CH}_2\text{OH, COEt, NH}_2, \text{phthalimide})\) is ascertained by several experimental techniques and their stability tested against diethylenetriaminepenta-acetate. Their photophysical properties (quantum yield, lifetime, radiative lifetime and sensitization efficiency) are presented and compared with those of the parent helicates \([\text{Ln}_2(\text{L}^3\text{C})_3]\). Finally, the cellular uptake of five Eu\(^{III}\) helicates is monitored by time-resolved luminescence microscopy and their localization in HeLa cells established by co-staining experiments.
1. Introduction

A key challenge in biology and medicine is to shed light on the structural and functional properties of cells, organs and living organisms. Imaging techniques are crucial tools towards this goal, and the availability of trustable experimental methods has profoundly influenced the development of modern medicine during the past 30 years. In particular, cancer detection and treatment necessitate sensitive and specific diagnosis and follow-up. Despite the panoply of high-tech imaging tools available, there is still much space for improvements, especially when it comes to spatial resolution, sensitivity and penetration depth. Present efforts focus on several aspects including combining drug delivery with a diagnostic agent (theranostics; [1]), and molecular imaging, which aims at gaining in vivo target-specific information with high sensitivity by means of probes mixing different modalities [2].

In any case, optical imaging, particularly luminescence imaging, is emerging as a vital component of the various tools required to meet the stringent requirements of modern medicine and biology [3]. A majority of luminescent labels are organic luminophores, which are highly emissive but which convey some major shortcomings: (i) they are often subject to intense photobleaching and some of them are operative for only a few seconds, (ii) their absorption and emission bands are overlapping (small Stokes’ shifts), (iii) background fluorescence interferes substantially, reducing their sensitivity/specificity, and (iv) their short excited state lifetimes make time-resolved detection technically quite involved. It is therefore understandable that alternatives are being sought. Semi-conductor quantum dots [4] are a possibility, but they are still not ideal because of emission flickering and concern about their toxicity [5]. Trivalent lanthanide ions represent other viable substitutes in view of their remarkable optical properties [6], enabling easy spectral and time discrimination and their low propensity to photobleaching.

Their only drawback is that part of these bioprobes are coordination compounds that are subject to dissociation in solution and in biological media. Therefore, adequate attention has to be devoted to the stability of these stains particularly if in vivo experiments are to be conducted. Lanthanide luminescent bioprobes, either coordination compounds [7] or nanoparticles [8,9], and their bioconjugates have been in use for more than 25 years in time-resolved immunoassays [10,11] with remarkable success, and they are now being thoroughly tested for bioimaging applications [12–14]. In this context, we have developed a new class of chelates based on the following concepts: (i) easy self-assembly in water under physiological conditions, (ii) large thermodynamic stability and kinetic inertness, (iii) adequate photophysical properties, (iv) low cytotoxicity, and (v) facile bioconjugation to bovine serum albumin, streptavidin (SA) or antibodies [15]. In addition, the chelates are dinuclear to allow, in the future, for bimodality, and their structure is helical for potential chiral assays.

The ditopic, hexadentate ligands (scheme 1) feature two compartments linked together by a methylene bridge and are based on a dipicolinic acid moiety derivatized in the 6-position by a benzimidazole unit acting as an efficient chromophore. They are also fitted with short polyoxyethylene pendants for better solubility and further chemical modification. They self-assemble with lanthanide ions under physiological conditions yielding highly stable [Ln2(L)3] helicates in which LnIII ions are 9-coordinate and well protected from solvent interaction. The initial chelates [Ln2(LCX)3](X = 2,3, see scheme 1) permeate several cancerous and non-cancerous cell lines, independently of the position of the polyoxyethylene arms [16,17]. The uptake mechanism is endocytosis, and luminescence from EuIII, TbIII and SmIII can be detected in time-resolved mode with large signal-to-noise ratios; moreover, no egress is observed after 24 h [18]. In addition, the [Eu2(LC2)3] helicate has been revealed to be a useful tool for the determination of DNA or RNA when associated with an organic dye such as acridine orange [19]. Several modifications have been implemented to explore the versatility of the new probes: lengthening of the polyoxyethylene arms [20], modification of the aromatic backbone to shift the excitation wavelength towards the visible in [Ln2(LC5)3]; [21], and substitution of the carboxylate coordinating units by phosphonate or phosphoester ones (e.g. H2LPOEt) [22]. Through all this work, H2LC2 emerged as being the best ligand, and suitable derivatization with carboxylic acid
groups to yield $H_4L^{C2COOH}$ allowed us to bioconjugate it to SA and monoclonal antibodies in order to develop very successful detection of cancerous cells in human breast tissue sections, owing to dual microfluidics assays of the receptors that they express [23].

In this paper, the extremity of the polyoxyethylene arms is functionalized with various groups in order to widen conjugation possibilities and, also, to test the influence of the substituent on cell intake and localization [24]. This in turn leads us to revisit the synthetic strategy for $H_2L^{C2}$ by inserting the substituent at the end of the synthetic route instead of during the initial steps. We therefore propose a new synthetic scheme for a common precursor $H_2L^{C2OH}$, which lends itself to further functionalizing by various methods. The resulting fine-tuning of the photophysical properties of the corresponding helicates is presented, as well as cell permeation investigations.

2. Results and discussion

(a) Novel synthesis route for $H_2L^{C2}$

The strategy successfully applied for the synthesis of $H_2L^{C_X}$ ($X = 1, 2, 5$) [17, 21, 25] consisted of a modified Phillips coupling reaction between the acyl chloride of a derivatized picolinic acid and 4,4'-methanediylbis(N-methyl-2-nitroaniline) (6, see scheme 2), to give a disubstituted product, followed by the formation of the bis(benzimidazole) rings under reductive conditions. However, the presence of the methoxy moiety on 4-methoxy-pyridine-2,6-dicarboxylic acid monoethyl ester 4 is detrimental to the overall reaction yield.

Alternatively, the reaction can be performed with 4 and 7 in phosphoric acid, or in polyphosphoric acid (PPA), as reported earlier [16], and here we have adopted a similar strategy. During the condensation step, the temperature has to be higher than 195°C but less than 230°C, otherwise the reaction mixture turns black and a brown solid is isolated, which has to be purified.
Scheme 2. Synthetic routes to the ditopic hexadentate ligands.

by chromatography on a Sephadex G25 column. In addition, it is important to ensure efficient stirring of the reaction, especially in the presence of PPA, which is a very viscous solvent. To limit the formation of polymeric by-products, due to the hydrolysis of the second carboxylic function of 4, diamine 7 is first dissolved at 195°C in PPA, then 4 is rapidly added to the solution.
before increasing the temperature to 205°C. This ensures rapid imine bond formation followed by cyclization. The brown solid consists of a mixture of products with the para position of the pyridines substituted by methoxy as well as hydroxyl substituents (calcd. 10% according to 1H NMR), due to partial hydrolysis of the methoxy protecting group under the harsh reaction conditions. Total deprotection of methoxy into hydroxyl groups is performed in acetic acid in the presence of hydrobromic acid, to yield $\text{H}_2\text{L}_{\text{C}_2\text{O}_2\text{H}}$ with a reasonable yield (30%, from 4). To limit the number of steps, we attempted to conduct the condensation step in PPA directly with 5, which bears a non-protected hydroxyl group on the pyridine 4-position, thus allowing ketone–enol equilibrium to occur. The target compound $\text{H}_2\text{L}_{\text{C}_2\text{O}_2\text{H}}$ was effectively isolated, but in low yield and in the presence of insoluble by-products, so that this idea was abandoned.

The last part of the synthesis deals with the conversion of the carboxylic acid moieties into ester ones, giving $\text{Et}_2\text{L}_{\text{C}_2\text{O}_2\text{H}}$, which is soluble enough into organic solvents to allow grafting of the arms. By using a Mitsunobu reaction with $\text{Et}_2\text{L}_{\text{C}_2\text{O}_2\text{H}}$ and $\text{H}(\text{OCH}_2\text{CH}_2)_3\text{OMe}$, the yield was low so that we turned to using brominated arm 8. The expected product $\text{Et}_2\text{L}_{\text{C}_2\text{O}}$ is obtained at a 75 per cent yield, and gives $\text{H}_2\text{L}_{\text{C}_2}$ as a pale yellow solid after hydrolysis. The overall yield is 19 per cent, which compares very favourably with the previous synthetic strategy (10%; [17]). The new synthetic procedure is also easier to perform and requires less purification steps. Above all, it allows inserting the arm during the final reaction steps, which makes it more appropriate than the previous route.

(b) Synthesis of the other ligands

Compared with $\text{H}_2\text{L}_{\text{C}_2}$, the presence of more hydrophilic end functions can induce different changes in the ligand properties: (i) we have demonstrated that the photophysical properties of tris(dipicolinate) derivatives can be fine-tuned depending on the nature of this group [26] and (ii) potentially, the different polarities of the resulting chelates may lead to different behaviours with respect to cell permeability. Therefore, differently substituted arms have been grafted on the $\text{Et}_2\text{L}_{\text{C}_2\text{O}_2\text{H}}$ precursor as follows. Firstly, a carboxylic acid substituent is introduced in view of further biocoupling suitable activation. The brominated reagent 9 is prepared in three steps, according to scheme 3, by protecting the carboxylic acid moiety 11 as an ester 12, followed by removal of the ether moiety to yield 13 and bromination of the alcoholic function to give 14. After reaction with $\text{Et}_2\text{L}_{\text{C}_2\text{O}_2\text{H}}$, the desired product $\text{Et}_2\text{L}_{\text{C}_2\text{O}_2\text{COH}}$ is isolated and subsequently converted into the tetra carboxylic ligand $\text{H}_4\text{L}_{\text{C}_2\text{COOH}}$. The $^{13}$C NMR spectra of the free ligand and of a 2 : 3 Lu$^{III}$ : L stoichiometric solution in D$_2$O (pD = 7.8) are presented in figure 1. Coordination of the lutetium cations induces chemical shifts for all C atoms and no resonance from the free ligand is observed, in line with the formation of the 2 : 3 dinuclear helicate [17]. In addition, the three ligands are equivalent on the NMR time scale, indicating a symmetrical environment within the

Scheme 3. Synthesis of the polyoxyethylene pendant arm.
complex. No decomplexation is observed after one week. We note that the \([\text{Eu}_2(\text{L}^{\text{C2COOH}})_3]^{3−}\) helicate has been previously coupled with avidin, yielding a luminescent bioconjugate \((Q = 9.3\%, \tau(5D_0) = 2.17 \text{ ms})\), which was successfully used as a detection probe for immunocyto- and immunohistochemical analyses [23].

Secondly, the polyoxyethylene arm was decorated with an amine function, which can also be used for bioconjugation. The amine is protected as a phthalimide, and deprotection occurs during the final step, together with the deprotection of both ester functions, to afford ligand \(\text{H}_2\text{LC2NH}_2\).

Finally, an alcohol group is fitted at the end of the arm by deprotecting \(\text{Et}_2\text{LC2}\) in the presence of TMSI \((N\text{-trimethylsilylimidazole})\) to yield \(\text{H}_2\text{LLC2CH2OH}\).

**Figure 1.** $^{13}$C NMR spectra of ligand \(\text{H}_4\text{L}^{\text{C2COOH}}\) (b) and of a stoichiometric 2 : 3 Lu$^{III}$ : ligand solution (a); \([\text{L}]_t = 8 \times 10^{-4} \text{ M in D}_2\text{O} (pD = 7.8)\).

(c) **Formation and stability of the [Ln$_2$(L$^\text{CX}$)$_3$] helicates**

Reaction of Ln$^{III}$ ions with the ditopic ligands shown in scheme 1 leads to the formation of [Ln$_2$(L$^\text{CX}$)$_3$] helicates as the major species in solution, and this is also the case for \(\text{H}_4\text{L}^{\text{C2COOH}}\), as previously demonstrated for Eu$^{III}$ by mass spectrometry [23] and confirmed for Lu$^{III}$ by $^{13}$C NMR (see above). In addition, luminescence titrations have been performed for both \(\text{H}_4\text{L}^{\text{C2COOH}}\) and \(\text{H}_2\text{L}^{\text{C2NH2}}\). As can been seen from **figure 2**, intensity of the Eu\((5D_0)\) luminescence reaches a maximum for \(\approx 0.67\) eq. of Eu$^{III}$, confirming the 2 : 3 stoichiometry of the helicates. The same result is obtained with the Tb$^{III}$ helicates (data not shown).

Furthermore, a competitive equilibrium experiment has been conducted by adding 100 equivalents of diethylenetriaminepentaacetate (DTPA) to a solution containing 2 : 3 stoichiometric amounts of Eu$^{III}$ and \(\text{H}_2\text{L}^{\text{C2NH2}}\). The intensity of the Eu\([5D_0 \rightarrow 7F_2]\) transition versus time is depicted on **figure 3**. One hour after the addition of the polyaminocarboxylate, the intensity had
Figure 2. Intensity of the Eu(\(5D_0\)) luminescence upon titration of \(\text{H}_4\text{L}^{\text{C}2\text{COOH}}\) (a) and \(\text{H}_2\text{L}^{\text{C}2\text{NH}_2}\) (b) with Eu\((\text{ClO}_4)_3\) in Tris–HCl 0.1 M (pH 7.4); \([L]_t = 3 \times 10^{-5}\) M and \(\lambda_{\text{ex}} = 320\) nm.

Figure 3. Time-course of the intensity of the Eu(\(5D_0 \rightarrow 7F_2\)) transition upon titrating \(\text{H}_2\text{L}^{\text{C}2\text{NH}_2}\) with Eu\(^{\text{III}}\) and adding 100 equivalents DTPA after reaching the stoichiometric 2 : 3 ratio. Solvent: Tris–HCl 0.1 M (pH 7.4), \([L]_t = 3 \times 10^{-5}\) M, \(\lambda_{\text{ex}} = 320\) nm.

decreased by less than 10 per cent. This shows that the \([\text{Eu}_2(\text{L}^{\text{C}2\text{NH}_2})_3]\) helicate is more stable than DTPA and kinetically rather inert, much as had been previously demonstrated for \([\text{Eu}_2(\text{L}^{\text{C}2})_3]\) [17]. Therefore, we may conclude that the functional groups at the end of the polyoxyethylene arms do not interfere with the formation of the helical complex and do not substantially modify its stability, as reported for tris(dipicolinates) bearing the same substituents [26].

(d) Luminescence properties of the \(\text{[Ln}_2(\text{L}^{\text{CX}})_3]\) helicates

The new hexadentate ligands sensitize the luminescence of several lanthanide ions in aqueous solution and pH 7.4. Representative emission spectra are shown in figure 4 for helicates with \(\text{H}_4\text{L}^{\text{C}2\text{COOH}}\), the spectra being almost identical for the other helicates. Spectra of helicates with La\(^{\text{III}}\), Gd\(^{\text{III}}\) and Lu\(^{\text{III}}\) only display a broad ligand-centred fluorescence band with a maximum around 25 000 cm\(^{-1}\), which is not present in the spectra of the Eu\(^{\text{III}}\) and Tb\(^{\text{III}}\) helicates. Instead, characteristic sharp \(f-f\) transitions from the Eu(\(5D_0\)) and Tb(\(5D_4\)) levels are seen. The Eu\(^{\text{III}}\) spectrum can be interpreted as arising from a major species having very similar chemical environments for the two metal ions with pseudo-D\(_3\) symmetry, by analogy with previously reported spectra for the helicates with ligands \(\text{H}_2\text{L}^{\text{CX}}\) \((X = 1–3, 5)\) and \(\text{H}_2\text{L}^{\text{POEt}}\) [15,21,22]. Indeed, the \(5D_0 \rightarrow 7F_0\) transition is very weak (it is forbidden in D\(_3\) symmetry) and reasonably
symmetrical, whereas the \( {^5D_0} \rightarrow {^7F_1} \) transition has two components, with the lower-energy one probably further split into a narrow doublet, reflecting a slight deviation from the idealized \( D_3 \) symmetry. Lifetime and quantum yield data are reported in Table 1.

All luminescence decays are single-exponential functions. The lifetime of the Eu\((^5D_0)\) level is between 2.23 and 2.45 ms, whereas \( \tau \)\((^5D_4)\) is shorter, lying within 0.60 and 0.75 ms. Both lifetimes are within the ranges observed for the other dinuclear helicates with similar \( N_6O_3 \) coordination environments. In the case of the Eu\(^{III} \) chelates, they reflect the absence of water interaction in the inner coordination sphere. The Eu\((^5D_0)\) lifetimes are the same, within experimental errors, as for helicates with \( H_2L^{C_2} \) and \( H_2L^{C_2COOH} \), while they are 8.5 per cent shorter for the other two chelates; a rational explanation is not obvious since the terminal functional groups in \( [Eu_2(L^{C_2CH_2OH})_3] \) and \( [Eu_2(L^{C_2NH_2})_3] \) that bear high-energy \( O^{-} \) and \( N^{-}H \) oscillators lie far from the emitting centre, unless the \( NH_2 \) and \( OH \) groups are involved in H-bonding with the coordinating carboxylates. No such shortening of the lifetime is seen for \( H_4L^{C_2COOH} \), probably because the helicate is under the anionic form \( [Eu_2(L^{C_2COO})_3]^{6-} \) at pH 7.4: indeed, the \( pK_a \) of the

**Figure 4.** Luminescence spectra of 2 : 3 stoichiometric solutions of Ln\(^{III} \) and \( H_4L^{C_2COOH} \) at room temperature; \([L] = 5 \times 10^{-4} \) M in Tris–HCl 0.1 M (pH 7.4), \( \lambda_{exc} = 320 \) nm.

**Table 1.** Ln\(^{III} \)(\( ^5D_0 \)) lifetimes and absolute quantum yields for 2 : 3 stoichiometric Ln : L solutions with \([L] = 5 \times 10^{-4} \) M in Tris–HCl 0.1 M (pH 7.4), \( \lambda_{exc} = 320 \) nm. Radiative lifetimes, intrinsic quantum yields and ligand sensitization efficacies are also given for Eu\(^{III} \).

<table>
<thead>
<tr>
<th>ligand</th>
<th>( Q_L^a ) (%)a</th>
<th>( \tau_{obs} (^{5D_0}) ) (ms)b</th>
<th>( \tau_{rad} (^{5D_0}) ) (ms)</th>
<th>( Q_L^a ) (%)d</th>
<th>( \eta_{sens} ) (%)</th>
<th>( Q_L^b ) (%)a</th>
<th>( \tau_{obs} (^{5D_4}) ) (ms)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>( H_2L^{C_2} )</td>
<td>21</td>
<td>2.43(9)</td>
<td>6.9</td>
<td>36</td>
<td>58</td>
<td>11</td>
<td>0.65(2)</td>
</tr>
<tr>
<td>( H_2L^{CCH_2OH} )</td>
<td>14.8</td>
<td>2.23(2)</td>
<td>7.6</td>
<td>29</td>
<td>51</td>
<td>14.8</td>
<td>0.75(1)</td>
</tr>
<tr>
<td>( H_2L^{C_2COOH} )</td>
<td>14.5</td>
<td>2.45(2)</td>
<td>6.1</td>
<td>40</td>
<td>36</td>
<td>6.9</td>
<td>0.75(1)</td>
</tr>
<tr>
<td>( H_2L^{C_2NH_2} )</td>
<td>2.3</td>
<td>2.23(1)</td>
<td>4.6</td>
<td>48</td>
<td>5</td>
<td>1.6</td>
<td>0.60(3)</td>
</tr>
</tbody>
</table>

\( \lambda_{exc} = 320 \) nm, ±10%.

\( \tau_{rad} \) for three determinations are given between parentheses.

\( n_{refractive} = 1.34; \) ±10%.

\( \eta_{sens} \) ±15%.

\( a \) From Chauvin et al. [17].
terminal carboxylic acid function should be similar to the one of 2-[2-(2methoxy-ethoxy)-ethoxy]acetic acid, 4.0 [27]. The Tb(5D4) lifetimes are shorter than expected for an N6O3 environment devoid of water molecules and this has been traced back for the previously reported helicates to a temperature-dependent back-transfer mechanism operating between 5D4 and the ligand triplet state [17].

Absolute quantum yields were determined by the integration sphere method [28]. They display a marked dependence with respect to the nature of the pendant arm as observed for tris(dipicolinate) derivatives bearing the same arms fitted with methoxy, alcohol and amine functions [26]. The replacement of the methoxy function in LC2 with an alcohol leads to a sizeable decrease in quantum yield, from 21 to 14.8 per cent for EuIII but to an increase for TbIII, from 11 to 14.8 per cent. The amine function has a highly detrimental effect on the quantum yields of both EuIII and TbIII helicates, which go down to 2.3 per cent (Eu) and 1.6 per cent (Tb), a tendency opposite to the one reported for the tris(dipicolinates) [26]. Finally, the terminal carboxylate induces a 36–37% decrease in the quantum yields of both EuIII and TbIII helicates. As said above, data for TbIII are difficult to interpret in view of the back-energy transfer process. For EuIII, the effects observed can be attributed to different influences by conducting a more detailed analysis. Indeed, quantum yields depend essentially on three parameters: (i) the sensitization efficiency $\eta_{\text{sens}}$ of the ligand, (ii) the relative importance of radiative and non-radiative processes at the metal centre, which is reflected in the observed lifetime, $\tau_{\text{obs}}$, and (iii) the radiative lifetime, $\tau_{\text{rad}}$, which depends on the refractive index and the nature of the coordination environment [29]. These parameters are linked by the following equations [6]:

$$Q_{\text{Ln}}^L = \eta_{\text{sens}} \times Q_{\text{Ln}}^L = \eta_{\text{sens}} \times \frac{\tau_{\text{obs}}}{\tau_{\text{rad}}}$$

in which $Q_{\text{Ln}}^L$ is the intrinsic quantum yield (i.e. the quantum yield upon direct excitation into the f-levels). In the case of EuIII, the radiative lifetime can be estimated from

$$\frac{1}{\tau_{\text{rad}}} = k_{\text{rad}} = A_{\text{MD},0} \times n^3 \times \frac{I_t}{I_{\text{MD}}}$$

where $A_{\text{MD},0} = 14.65 \text{s}^{-1}$ is the rate constant of the magnetic dipole transition $^5D_0 \rightarrow ^7F_1$, $n$ is the refractive index of the medium, and $I_t$ and $I_{\text{MD}}$ are the integrated emission areas of the $^5D_0 \rightarrow ^7F_j$ ($j = 0–6$) and $^5D_0 \rightarrow ^7F_1$ transitions, respectively. The corresponding parameters are reported in table 1. The observed lifetimes have little influence since they do not fluctuate much in the series. Similarly, the radiative lifetimes are comparable to that of the reference [Eu2(LC2)] helicate (within the ±10% experimental error), as expected in view of the identical coordination environments, except for [Eu2(LC2NH2)] helicate, for which an unexplained diminution of about one-third is found. As a consequence, the intrinsic quantum yield of this helicate is the largest, at 48 per cent. Although a favourable factor, this however does not translate into an improved overall quantum yield for [Eu2(LC2NH2)] helicate; on the contrary, the sensitization efficiency for this helicate is miserable, at only 5 per cent, resulting in a very low value of $Q_{\text{Ln}}^L$. The intrinsic quantum yield for [Eu2(LC2CH2OH)] is the smallest, at 29 per cent, a consequence of this helicate having the largest radiative lifetime (7.6 ms). On the other hand, these two parameters are very similar for [Eu2(LC2)] and [Eu2(LC2COOH)] helicates. The 30 per cent reduction in $Q_{\text{Ln}}^L$ for [Eu2(LC2CH2OH)] and [Eu2(LC2COOH)] helicates with respect to the chelate with H2LC2 has different origins. For the former chelate, the main actor lies in the small intrinsic quantum yield, whereas for the other complex, the decrease is essentially a consequence of the smaller sensitization efficiency. That is, despite their apparent long distance from the metal centre, the end substituents of the arms considerably modulate the lanthanide luminescence properties.

e) Cell permeability of the EuIII helicates

In principle, one may think that the global charge of a metal complex and/or its lipophilicity can drastically influence its cell cytotoxicity, permeability and localization. In our previous
studies though, all neutral helicates investigated showed the same type of endosomal/liposomal localization after endocytosis. Moreover, due to the large molecular weight of the bioprobes (around 2800–3200 Da), their egress is very slow [18]. Therefore, here we test the influence of: (i) the coordinating group, phosphonate in $[\text{Eu}_2(L\text{POE})_3]$ [22] versus carboxylate in $[\text{Eu}_2(L\text{C}_2\text{O}_2\text{H})_3]$, (ii) the polarity of the pendant arms, OH substituent in $[\text{Eu}_2(L\text{C}_2\text{CH}_2\text{OH})_3]$ versus OMe terminal function in $[\text{Eu}_2(L\text{C}_2\text{O}_2)_3]$), and/or (iii) the overall charge of the probe by using a negatively charged complex $[\text{Eu}_2(L\text{C}_2\text{COOH})_3]^{6-}$ or a potentially positively charged one $[\text{Eu}_2(L\text{C}_2\text{NH}_2)_3]^{6+}$; indeed, the pH of some endosomal compartments can reach values as low as 2 and because the $pK_a$ of the terminal amine is around 7.0, it is expected that it will be protonated at such a low pH value. The phosphonate helicate $[\text{Eu}_2(L\text{POE})_3]$ was chosen in place of the polyoxyethylene-fitted $[\text{Eu}_2(L\text{POE})_3]$ (scheme 1) because its quantum yield is much larger (25% versus only 2.5%; [22]).

All the helicates bearing different coordinating and terminal groups permeate into HeLa cells and stain the cytoplasm. After cellular uptake, time-resolved microscopy images show the complexes being present in distinct vesicles in the cytoplasm of the chelate-loaded cells. This finding points to an endocytotic mechanism for cellular uptake, as expected. The localization is confirmed by the use of LysoTracker Blue, a fluorescent marker for secondary endosomes and lysosomes. When HeLa cells are simultaneously incubated with the Eu$^{III}$ helicates and the organic marker, the superimposed images (figure 5) show a substantial overlap (greater than 60%) of the

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**Figure 5.** Co-localization imaging of HeLa cells loaded with 100 µM helicates ($[\text{Eu}_2(L\text{C}_2\text{O}_2\text{H})_3]$, $[\text{Eu}_2(L\text{C}_2\text{COOH})_3]$, $[\text{Eu}_2(L\text{POEt})_3]$ or 200 µM $[\text{Eu}_2(L\text{C}_2\text{NH}_2)_3]$) and 500 nM LysoTracker Blue DND-22 together for 4 h at 37°C. (a) Bright field; (b) Eu$^{III}$ luminescence recorded in time-resolved mode; (c) fluorescence of LysoTracker Blue DND-22; and (d) merged image. See §2e for details.
red Eu\textsuperscript{III} luminescence with the blue fluorescence of the LysoTracker Blue dye. In summary, we conclude that most helicates are present in secondary endosomes and lysosomes, irrespective of their polarity or overall charge. A similar conclusion has been reached for cyclen-based lanthanide bioprobes \cite{30–32}.

3. Conclusion

The new route proposed for the synthesis of ditopic ligands derived from H\textsubscript{2}L\textsuperscript{C\textsubscript{2}} is a real improvement in that it is simpler to carry out and leads to almost twofold increased yields. In turn, this permits testing the effects of several different substitutions of the extremity of the polyoxyethylene arms on the photophysical and cell-uptake properties of the helicates. As a matter of fact, experimental data show that if coordination properties are not much affected, the radiative lifetimes and more importantly the quantum yields of the [Ln\textsubscript{2}(L\textsuperscript{C\textsubscript{2}}\textsuperscript{X})\textsubscript{3}] helicates (Ln = Eu, Tb; X = C\textsubscript{2}, C\textsubscript{2}COOH, C\textsubscript{2}CH\textsubscript{2}OH and C\textsubscript{2}NH\textsubscript{2}) are modulated by the end substituent. Conversely, if all tested Eu\textsuperscript{III} complexes are suitable as cell-imaging probes, the ability of the chelates to permeate cells is not modified compared with [Eu\textsubscript{2}(L\textsuperscript{C\textsubscript{2}}\textsuperscript{X})\textsubscript{3}], even if the complexes bear different global charges.

In a succession of seminal studies, Parker and co-workers \cite{30,31,33,34} have investigated a series of 60 macrocyclic complexes based on the cyclen framework and fitted with various chromophores such as tetraazaphenylene, acridone, azaxanthone, azathiaxanthone or pyrazolyl azaxanthone as sensitizing units. They obtained radically different results for the cytotoxicity, cell permeation and localization of the luminescent stains by modifying the substituents on the cyclen-type ligands \cite{31}. About 80 per cent of the chelates have endosomal/lysosomal localization and are non-toxic, whereas the remaining complexes have larger cytotoxicity (IC\textsubscript{50} < 90 \mu M) and locate either in mitochondria or in the nuclei. In fact, these investigations have shown that the cell-intake properties of the chelates do not depend on their overall charge or lipophilicity; only on the nature of the substituent; the reason is that polyaromatic substituents are recognized by protein association. In our case, all substituents are aliphatic so that this discriminating recognition factor does not exist. The data presented here therefore confirm that cell intake and localization are independent of the charge of the complex and of its polarity/lipophilicity.

4. Experimental section

(a) Starting materials and general procedures

Chemicals and solvents were purchased from Fluka A.G. Solvents were purified by a non-hazardous procedure by passing them onto activated alumina columns (Innovative Technology Inc. system; \cite{35}). Previously reported syntheses include 4-hydroxy-pyridine-2,6-dicarboxylic acid diethyl ester 2 from chelidamic acid 1 (yield: 75%; \cite{16,36}), 3,3′-dinitro-4,4′-bis-(N-aminomethyl-diphenyl)methane 6 (yield: 75%) and 4,4′-methanediyl-bis(N-methylbenzene-1,2-diamine) 7 (yield: 85%) \cite{16,22,37}, H\textsubscript{2}L\textsuperscript{POEt} \cite{22}, Et\textsubscript{2}L\textsuperscript{C\textsubscript{2}} and H\textsubscript{2}L\textsuperscript{C\textsubscript{2}} \cite{17}, the phthalimide-fitted arm 10 \cite{26}, as well as the bromination of 2-[2-(2-methoxy-ethoxy)-ethoxy]-ethanol with PBr\textsubscript{3} to yield arm 8 \cite{38,39}, see scheme 2 for the numbering of the compounds.

(b) Analytical measurements

NMR spectra were measured at 25°C on Bruker Avance DRX 400 (\textsuperscript{1}H, 400 MHz) and AV 600 (\textsuperscript{13}C, 99.8 MHz) spectrometers. Spectra were recorded in CDCl\textsubscript{3} (99.8%, Aldrich), (MeOD (99.8%, Aldrich) or deuterated dimethylsulfoxide (DMSO-\textit{d}_{6}; 99.8% Aldrich); deuterated solvents were used as internal standards, and chemical shifts \(\delta\) (ppm) are given with respect to TMS. The ESI-MS spectra of the ligands were obtained on a Finningan SSQ 710C spectrometer using \(10^{-5}\) to \(10^{-4}\) M solutions in acetonitrile/H\textsubscript{2}O/acetic acid (50/50/1) or MeOH, capillary temperature 200°C and acceleration potential 4.5 keV. The instrument was calibrated using the horse myoglobin standard,
and analyses were conducted in positive mode. ESI-QT of MS spectra of the complexes were measured on a Q-Tof Ultima API mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray type ESI source. Phosphoric acid was used for the positive ion mass calibration range of 100–2000 m/z. Data were acquired and processed using MASSLYNX v. 4.0. Electrospray conditions were as follows: capillary voltage, 2.3 kV; source temperature, 80°C; cone voltage, 35 V; and source block temperature, 150°C. The ESI nebulization and drying gas was nitrogen. All experiments were performed in positive ion mode. The sample was introduced via a syringe pump operating at 20 µl min⁻¹. Simulation of spectra was achieved with MOLECULAR WEIGHT CALCULATOR v. 6.42. Elemental analyses were performed by Dr Solari, at the École Polytechnique Fédérale de Lausanne.

(c) 4-Methoxy-pyridine-2,6-dicarboxylic acid diethyl ester (3)

This synthesis was adapted from Horvath et al. [40]. 4-Hydroxy-pyridine-2,6-dicarboxylic acid diethyl ester 2 (2.4 g, 1 mmol) was dissolved in dimethylformamide (DMF), in the presence of K₂CO₃ (2.2 g); Me₂SO₄ was added to the solution, which was stirred at RT for 2 h. The solvents were evaporated under vacuum, the product was dissolved in dichloromethane, washed with half-saturated solution of aqueous NH₄Cl, dried over Na₂SO₄ and the solvent removed, giving 2.4 g of the desired product 3 (98%). Anal. calcd. for C₁₂H₁₅NO₅: (%): C 56.91, H 5.97, N 5.53; found: C 56.92, H 5.97, N 5.55. ¹H NMR (CDCl₃, ppm): 7.79 (s, 1 H, Har), 4.47 (q, J = 7.0 Hz, 2 H, −CH₂−), 1.45 (t, J = 7.0 Hz, 3 H, −CH₃).

(d) 4-Methoxy-pyridine-2,6-dicarboxylic acid monoethyl ester (4)

A solution of NaOH (220 mg, 5.5 mmol) in 50 ml of ethanol was added to 3 (1.4 g, 5.5 mmol) in ethanol (50 ml), and the resulting solution was stirred at RT for 2 h. The evolution of the reaction was followed by TLC (silica plate, CH₂Cl₂/MeOH 97/3 v/v). After completion of the reaction, the solution was evaporated and the product dissolved in water. The basic aqueous solution was washed with 2 × 50 ml CH₂Cl₂, then the pH of the aqueous phase was decreased until precipitation of a pale yellow solution (pH = 1.8), which was collected, washed with water and dried. Yield: 1 g, 84 per cent. ESI-MS: m/z = 226.26 [M + H]⁺ (calcd. 226.06). ¹H NMR (400 MHz, CDCl₃, ppm): 7.86 (d, J = 2.6 Hz, 1 H, Har), 7.82 (d, J = 2.6 Hz, 1 H, Har), 4.47 (q, J = 7.3 Hz, 2 H, −CH₂−), 1.44 (t, J = 7.3 Hz, 3 H, −CH₃).

(e) 4-Hydroxy-pyridine-2,6-dicarboxylic acid monoethyl ester (5)

This compound was synthesized similarly to the procedure reported for 4, giving the desired product as a pale yellow solid in 80 per cent yield. ESI-MS: m/z = 212.37 [M + H]⁺ (calcd. 212.06). ¹H NMR (400 MHz, DMSO-d₆, ppm) 7.56 (s, 2 H, Har), 4.35 (q, J = 7.0 Hz, 2 H, −CH₂−), 1.33 (t, J = 7.0 Hz, 3 H, −CH₃).

(f) Et₂L²⁰Me

The synthesis was performed with a modified Phillips coupling reaction, starting from 4 (450 mg, 2 mmol) and followed by reduction in the presence of iron [17]; 190 mg were collected (yield: 30% over two steps). ESI-MS: m/z = 318.30 [M + 2H]⁺/2 (calcd. 318.35). ¹H NMR (400 MHz, CDCl₃, ppm): 8.13 (d, J = 2.3 Hz, 1 H, Har), 7.73 (s, 1 H, Har), 7.70 (d, J = 2.6 Hz, 1 H, Har), 7.38 (m, 1 H, Har), 7.26 (dd, J₁ = 8.5 Hz, J₂ = 1.8 Hz, 2 H, Har), 4.50 (q, J = 7.0 Hz, 2 H, −OCH₂−), 4.40 (s, 3 H, −OCH₃), 4.32 (s, 1 H, −CH₂−), 4.04 (s, 3 H, −NCH₃), 1.48 (t, J = 7.3 Hz, 3 H, −CH₃).

(g) H₂L²⁰Me

A solution of sodium hydroxide (20 mg, 0.5 mmol) in 5 ml EtOH was added to a solution of 8 (160 mg, 0.25 mmol) dissolved in ethanol/water (15 ml). The solution was stirred at RT for 2 h then
the solvents were removed, the compound dissolved in water and washed with dichloromethane (2 × 25 ml). The pH was adjusted to 2 and a precipitate formed, which was collected, rinsed with water and dried under vacuum to provide H₂L₄C₂OH as a yellow solid (yield: 80%). Anal. calcd. for H₂L₄C₂OMe · 2 HCl (C₃₁H₂₆N₆O₆ · 2HCl): C 51.73, H 4.34, N 11.68; found: C 51.70, H 4.29, N 11.49. ¹H NMR (400 MHz, DMSO-d₆, ppm): 7.65 (d, J = 2.6 Hz, 1 H, Har); 7.58 (d, J = 0.6 Hz, 1 H, Har), 7.53 (s, 1 H, Har), 7.48 (d, J = 2.3 Hz, 1 H, Har), 7.23 (m, 1 H, Har), 4.26 (s, 3 H, −OCH₃), 4.21 (s, 1 H, −CH₂−), 3.91 (s, 3 H, −NCH₃).

(h) H₂L₄C₂OH

Freshly prepared 4,4′-methanediylbis(N-methylbenzene-1,2-diamine) 7 (1.4 g, 5.5 mmol) was introduced into PPA (20 ml) and the mixture was heated with a DrySyn apparatus at 195 °C; compound 4 (2.7 g, 12.2 mmol) was added as a solid to the brown viscous mixture and the temperature increased to 205 °C. After stirring for 5 h, the temperature was decreased to approximately 60 °C and the viscous brown residue poured into 100 ml of water. A solid formed, which was filtered, dissolved in NH₄OH 25 per cent and precipitated again in the presence of HCl 25 per cent. The solid was collected, rinsed with water, poured into acetic acid (15 ml) and the solution was refluxed for 5 h. The solvents were removed and the solid dried under vacuum. The resulting compound (1.4 g, 45%) can be used as such or purified with a Sephadex G25 column (eluent: water). Alternatively, Et₂L₄C₂OMe (200 mg) was converted into H₂L₄C₂OH in acetic acid (5 ml) in the presence of HBr 47 per cent (1.4 ml) and the solution was refluxed for 5 h. The solvents were removed and the solid dried under vacuum. The resulting compound (1.4 g, 45%) can be used as such or purified with a Sephadex G25 column (eluent: water). Alternatively, Et₂L₄C₂OMe (200 mg) was converted into H₂L₄C₂OH in acetic acid (5 ml) in the presence of HBr 47 per cent (0.4 ml), as described above (yield: 80%). Anal. calcd. for H₂L₄C₂OH · MeCO₂H · H₂O (C₂₉H₂₂N₆O₆ · C₂H₄O₂ · H₂O): C 60.00, H 4.40, N 13.50; found: C 60.26, H 4.59, N 13.97. ESI-MS m/z = 551.38 [M + H]⁺ (calcld. 551.16); 276.45 [M + 2H]⁺/2 (calcld. 276.09). ¹H NMR (400 MHz, D₂O, ppm): 8.10 (m, 1 H, Har), 7.94 (m, 1 H, Har), 7.76 (s, 1 H, Har), 7.48 (s, 1 H, Har), 7.37 (m, 1 H, Har), 4.66 (m, 1 H, −CH₂−), 4.31 (s, 3 H, −NCH₃).

(i) Et₂L₂C₂OH

Compound H₂L₂C₂OH (140 mg, 0.51 mmol) was refluxed in ethanol (100 ml) in the presence of sulfuric acid (2 ml) for 5 h, then the solution was poured into a saturated NaHCO₃ solution. A precipitate formed, which was collected, rinsed with water then with ethanol and dried under vacuum (130 mg, yield 90%). Anal. calcd. for C₃₂H₃₀N₆O₆ · 2.15 H₂O: C 59.82, H 5.19, N 12.61; found: C 59.85, H 4.99, N 12.42. ¹H NMR (400 MHz, DMSO-d₆, ppm): 7.69 (m, 1 H, Har), 7.58 (s, 1 H, Har), 7.52 (d, J = 8.2 Hz, 1 H, Har), 7.35 (m, 1 H, Har), 7.25 (d, J = 8.2 Hz, 1 H, Har), 4.33 (q, J = 7.0 Hz, 4 H, −OCH₂−), 4.27 (s, 3 H, −NCH₃), 4.18 (s, 1 H, −CH₂−), 1.33 (t, J = 7.0 Hz, 6 H, −CH₃). ¹³C NMR (DMSO-d₆, ppm): 166.5 (C = O), 165.0 (C = O), 164.8 (Car − O), 152.0 (Car), 149.5 (Car), 148.7 (Car), 142.6 (Car), 136.9 (Car), 136.2 (Car), 125.0 (Car), 119.5 (Car), 114.2 (Car), 113.4 (Car), 111.1 (Car), 61.7 (OCH₂), 60.8 (OCH₂), 41.8 (CH₂), 33.1 (N − CH₃).

(j) H₂L₄C₂CH₂O₂H

TMSI (0.12 ml, 0.88 mmol) was added dropwise to a solution of Et₂L₂C₂ (200 mg, 0.22 mmol) dissolved in MeCN (50 ml). The solution was heated at 70°C for 2 h under a nitrogen atmosphere, then left to return to RT. Methanol was added (20 ml) and the solvents were evaporated. The crude product was re-dissolved in dichloromethane and washed with an aqueous solution of Na₂S₂O₃ 0.5 M. The organic phase was dried over Na₂SO₄ and evaporated. The product was directly hydrolyzed, as described above. The crude product was purified by chromatography (reverse phase, MeCN, H₂O, TFA 0.05%) to give H₂L₄C₂CH₂O₂H as a pale yellow solid (120 mg, yield 65% over two steps). Anal. calcd. for C₄₁H₄₆N₆O₁₂ 1.5 TFA 1.8 H₂O: C 48.38, H 4.72, N 7.69; found: C 48.33, H 4.58, N 7.56. ¹H NMR (600 MHz, MeOD): 8.09 (d, J = 2.18 Hz, 2H, Har), 7.93 (d, J = 2.18 Hz, 2H, Har), 7.88 (s, J = 8.55 Hz, 2H, Har), 7.53 (d, J = 8.55 Hz, 2H, Har), 7.50 (d, J = 8.55 Hz, 1H, Har), 4.44 (d, J = 4.37 Hz, 4H, −OCH₂−), 4.38 (2H, −CH₂−), 4.34 (6H,
NCH3), 3.95 (t, J = 4.37 Hz, 4H, −OCH2−), 3.73 (d, J = 6.4 Hz; d, J = 5.0 Hz; 4H, −OCH2−), 3.63 (d, J = 6.4 Hz; d, J = 5.0 Hz; 4H, −OCH2−), 3.64 (d, J = 6.4 Hz; d, J = 5.0 Hz; 4H, −OCH2−), 3.58 (d, J = 6.4 Hz; d, J = 5.0 Hz; 4H, −OCH2−), 3.55 (s, 6H, OCH3). 13C NMR (600 MHz, D2O): 168.76 (C=O), 167.21 (C=O), 151.70 (C=O), 149.44 (C=O), 148.68 (C=O), 140.63 (C=O), 137.70 (C=O), 135.56 (C=O), 128.19 (C=O), 120.21 (C=O), 116.90 (C=O), 115.11 (C=O), 114.34 (C=O), 113.09 (C=O), 73.69 (OCH2), 71.86 (OCH2), 71.47 (OCH2), 70.36 (OCH2), 69.90 (OCH2), 62.19 (OCH3), 49.14 (CH2), 42.66 (N−CH3).

(k) [2-(2-Methoxy-ethoxy)-ethoxy]-acetic acid ethyl ester (12)

[2-(2-Methoxy-ethoxy)-ethoxy]-acetic acid 11 (1.8 g, 10 mmol) was refluxed for 4 h in the presence of sulfuric acid (2 mol), then the solvent was removed and the solution was poured into a saturated NaHCO3 solution. The pH was adjusted to 7–8, and the solution was extracted with 3 × 100 ml of dichloromethane. The organic phase was dried over Na2SO4 and the solvent removed to give the desired product as a colourless oil (2.0 g, yield 97%). 1H NMR (400 MHz, CDCl3, ppm): 4.21 (q, J = 7.3 Hz, 2 H, −OCH2−CH3), 4.15 (s, 2 H, −CH2−CO), 3.75 (m, 2 H, −CH2−), 3.70 (m, 2 H, −CH2−), 3.65 (m, 2 H, −CH2−), 3.55 (m, 2 H, −CH2−), 3.38 (s, 3 H, −OCH3), 1.28 (m, 3 H, −CH3).

(l) [2-(2-Hydroxy-ethoxy)-ethoxy]-acetic acid ethyl ester (13)

Compound 12 (1 g, 4.8 mmol) was refluxed in dry acetonitrile (50 ml) for 2 h with iodotrimethylsilane (19 mmol), then ethanol was added (20 ml) and the solvents were evaporated. The brown residue was dissolved in dichloromethane (50 ml) and stirred with an aqueous solution of Na2S2O3 (7.5 g in 50 ml of water) until the solution turned bright yellow. The aqueous phase was extracted with 3 × 50 ml of dichloromethane, the organic phases dried over Na2SO4 and the solvents removed to afford 13 as a pale yellow compound (0.9 g, yield 95%). ESI-MS m/z calcd. for [M + Na]+ (found): 215.21 (215.27). 1H NMR (400 MHz, CDCl3, ppm): δ = 4.22 (m, 2 H, −CH2−), 4.14 (s, 2 H, −CH2−CO), 3.72 (m, 6 H, −CH2−), 3.62 (m, 2 H, −CH2−), 1.28 (t, J = 7.0 Hz, 3 H, −CH3).

(m) Synthesis of 14

The bromation of 13 was performed similarly to the one reported for 8, starting from 1 g [5.2 mmol] and gave the desired product as an oil (0.5 g, yield 33%). 1H NMR (400 MHz, CDCl3, ppm): 4.22 (q, J = 7.0 Hz, 2 H, −CH2−), 4.18 (s, 2 H, −CH2−CO), 3.64 (t, 3 J = 4.55 Hz, 2 H, −CH2−), 3.73 (m, 4 H, −CH2−), 3.48 (t, 3 J = 4.55 Hz, 2 H, −CH2−), 1.29 (t, J = 7.0 Hz, 3 H, −CH3).

(n) Et2L2C2OEt2

The synthesis was performed as reported for Et2L2C2OH, starting from Et2L2C2OH (250 mg, 0.4 mmol) in 10 ml of DMF and 15 (245 mg, 0.9 mmol). Et2L2C2OEt2 was isolated as a yellow product and was used without purification in the next step. ESI-MS m/z calcd. for [M + 2H]+/2 (found): 478.21 (478.38). 1H NMR (400 MHz, CDCl3): 8.07 (d, J = 2.3 Hz, 1 H, Hαr), 7.69 (d, J = 2.3 Hz, 1 H, Hαr), 7.60 (s, 1 H, Hα), 7.33 (d, J = 8.5 Hz, 1 H, Hαr), 7.15 (d, J = 8.5 Hz, 1 H, Hαr), 4.44 (q, J = 7.0 Hz, 2 H, OCH2−CH3), 4.25 (s, 1 H, −COCH2−), 3.90 (m, 1 H, −CH2−), 3.80 (t, J = 6.4 Hz, 4 H, −OCH2−), 3.61 (m, 2 H, −OCH2−), 3.54 (m, 2 H, −OCH2−), 3.46 (m, 2 H, −OCH2−), 3.37 (s, 3 H, NCH3), 1.43 (t, J = 7.0 Hz, 3 H, OCH2−CH3), 1.24 (t, 3 H, OCH2−CH3).

(o) H2L2C2COOH

Et2L2C2OEt2 (100 mg, 0.11 mmol) was hydrolyzed in EtOH/H2O (50/50 v/v, 50 ml) in the presence of NaOH (28 mg, 0.67 mmol). The evolution of the reaction was monitored by TLC
(silica, CH₂Cl₂/MeOH 97/3 v/v). The solution was washed by CH₂Cl₂ (3 × 50 ml), then the pH was adjusted to 2 by HCl 1 M, from which H₂L₂COOH precipitated. It was collected, rinsed with cold water and dried under vacuum to give a brown solid (96 mg, yield 80%). Anal. calcd. for C₄₄H₄₂N₆O₁₄·HCl·0.25 H₂O: C 55.72, H 4.96, N 9.51; found: C 55.76, H 4.82, N 9.79. ESI-MS m/z calcd. for [M + 2H⁺]/2 (found): 422.14 (422.35). ^1H NMR (400 MHz, D₂O, NaOD 0.05 M): 7.45 (d, J = 2.9 Hz, 2 H, Har), 7.26 (m, 2 H, Har), 7.04 (m, 1 H, Har), 4.23 (m, 2 H, −OCH₂− and −CH₂−), 3.92 (m, 5H, −OCH₂− and −NCH₃), 3.77 (m, 2 H, −OCH₂−), 3.69 (m, 2H, −OCH₂−). ^13C NMR (125 MHz, H₂O, NaOD 0.05 M): 178.0 (C=O), 172.0 (C=O), 166.0 (Car−O), 155.5 (Car), 150.2 (Car), 149.4 (Car), 140.9 (Car), 137.3 (Car), 134.8 (Car), 125.0 (Car), 117.7 (Car), 111.9 (Car), 110.8 (Car), 110.6 (Car), 69.8 (OCH₂), 69.4 (OCH₂), 68.6 (OCH₂), 67.4 (OCH₂), 41.3 (CH₂), 32.0 (N−CH₃).

(p) H₂L₂C₂NH₂

The synthesis was performed analogously to that of Et₂L₂C₂COOH, starting from Et₂L₂C₂OH (200 mg, 0.11 mmol) and HO(CH₂CH₂)₃Phta (95 mg, 0.33 mmol). Et₂L₂C₂Phta was isolated as a brown product, which was immediately hydrolyzed in the presence of an excess of NaOH 5 M (15 ml), and heated at 90 °C for 12 h. The basic aqueous solution was then washed with 2 × 50 ml CH₂Cl₂, and the pH of the aqueous phase was adjusted to 1.5. A solid precipitated, which was collected, rinsed with water and dried under vacuum to afford H₂L₂C₂NH₂ as a pale yellow solid (50 mg, yield 55%). Anal. calcd. for C₄₁H₄₈N₈O₁₀·HCl·0.5 H₂O: C 57.37, H 5.87, N 13.06; found: C 57.27, H 5.38, N 12.83. ^1H NMR (400 MHz, D₂O): 7.95 (m, 2 H, Har), 7.45 (m, 2 H, Har), 7.27 (m, 1 H, Har), 4.36 (m, 2 H, −CH₂−), 4.28 (m, 3 H, −NCH₃), 3.72–3.43 (m, 12H, −OCH₂−). ^13C NMR (125 MHz, D₂O) δ (ppm): 178.3 (C=O), 172.1 (C=O), 166.9 (Car−O), 154.9 (Car), 150.8 (Car), 149.1 (Car), 140.0 (Car), 138.2 (Car), 134.1 (Car), 125.9 (CH₂), 116.7 (CH₂), 113.1 (CH₂), 111.2 (CH₂), 111.0 (CH₂), 68.5 (OCH₂), 69.0 (OCH₂), 67.6 (OCH₂), 67.0 (OCH₂), 41.0 (CH₂), 31.5 (N−CH₃).

(q) Preparation of the lanthanide helicates

The 2 : 3 complexes were synthesized in situ by mixing stoichiometric quantities of H₂L₂C₂X and Ln(ClO₃)₄·x H₂O (Ln = Eu, Tb, x = 2.5–4.5) in water. These salts were prepared from their oxides (Rhône–Poulenc, 99.99%) in the usual way [41]. Concentration of the perchlorate solutions was determined by complexometric titrations with a standardized Na₂H₂ ethylenediaminetetraacetic acid solution in urotropine-buffered medium and with xylene orange as the indicator [42].

(r) Luminescence measurements

Luminescence spectra and lifetimes (three independent measurements) were collected on a Horiba-Jobin Yvon Fluorolog FL-3–22 fluorimeter using 0.1 cm quartz Suprasil cuvettes. All spectra were corrected for the experimental function of the instrument. Quantum yields were measured by an absolute method using an integration sphere [28] and are given as averages of three independent measurements.

(s) Cell line and culture conditions

The human cervical adenocarcinoma cell line HeLa (ATCC CCL-2) was used in this study. Cells were cultivated in 25 cm² culture flasks using RPMI 1640 (Sigma, R8758, UK) supplemented with 5 per cent foetal calf serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 1 per cent non-essential amino acids, 1 per cent 4-(2-hydroxyethyl)-monosodium salt (all from Gibco Cell Culture, Invitrogen, Basel, Switzerland). Cultures were maintained at 37°C under a 5 per cent CO₂ and 95 per cent air atmosphere. The growth medium was changed every other day until the time of use of the cells. Cell density and viability, defined as the ratio of the number of viable
cells over the total number of cells, of the cultures were determined by trypan blue staining and a Neubauer improved haemacytometer (Blau Brand, Wertheim, Germany).

(t) Cell imaging
To detect the localization of the helicates, HeLa cells were seeded on a plastic bottom µ-dish (ibidi GmbH), incubated with helicates (100 µM [Eu₂(LC₂)]₃, [Eu₂(LC₂CH₂OH)]₃, [Eu₂(LC₂COOH)]₃⁵⁻, [Eu₂(LP₂Oβ)]₃ or 200 µM [Eu₂(LC₂NH₂)]₃) and 500 nM LysoTracker Blue DND-22 at 37°C and 5 per cent CO₂ for 4 h. Cells were examined with a home-modified time-resolved luminescence setup Signifier from Wallac Oy equipped with a Nikon Eclipse 600 microscope and an ORCA-ER CCD camera (Hamamatsu). The luminescence of helicates within the cells was detected in time-resolved mode, and the fluorescence of the LysoTracker Blue dye was measured with conventional fluorescence microscopy. The following measurement conditions were used: (i) for time-resolved luminescence imaging, objective: Plan-fluor 100×, excitation: bandpass filter 340 nm (BP 70 nm), emission: long-pass filter 420 nm, delay time: 100 µs, exposure time: 30 s; (ii) for fluorescence imaging, objective: Plan-fluor 100×, excitation: bandpass filter 365 nm (BP 80 nm), emission: bandpass filter 450 nm (BP 65 nm), exposure time: 8 s.

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