Lanthanides

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Bright, Highly Water-Soluble Triazacyclononane Europium Complexes To Detect Ligand Binding with Time-Resolved FRET Microscopy**

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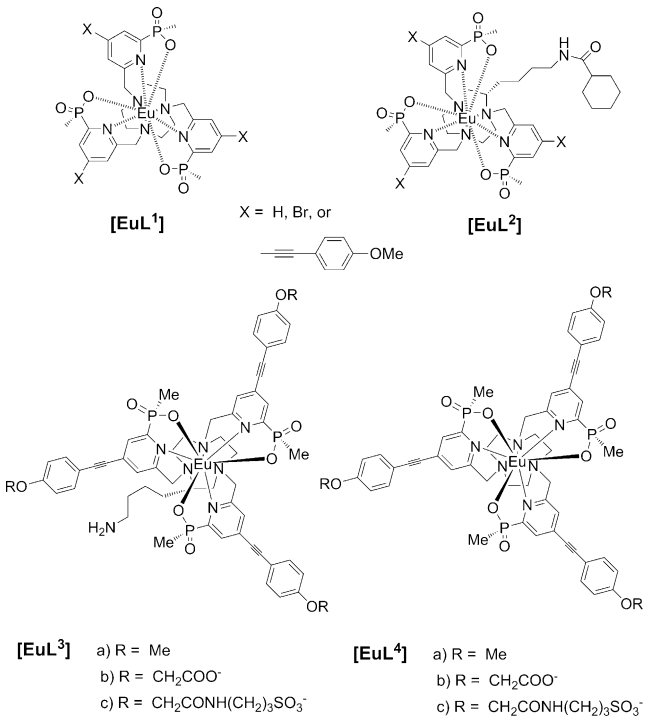
Abstract: *Luminescent europium complexes are used in a broad range of applications as a result of their particular emissive properties. The synthesis and application of bright, highly water-soluble, and negatively charged sulfonic- or carboxylic acid derivatives of para-substituted aryl–alkynyl triazacyclononane complexes are described. Introduction of the charged solubilizing moieties suppresses cellular uptake or adsorption to living cells making them applicable for labeling and performing assays on membrane receptors. These europium complexes are applied to monitor fluorescent ligand binding on cell-surface proteins with time-resolved Förster resonance energy transfer (TR-FRET) assays in plate-based format and using TR-FRET microscopy.*

Luminescent lanthanide complexes are used in an impressive number of applications, such as sensors,[1,2] bioassays,[3] or OLEDs (organic light-emitting diodes),[4] because of their peculiar photophysical properties. Their use in luminescence-based cellular imaging has been particularly interesting, as background fluorescence from cells can be completely rejected using time gating.[1,2,5,6] A key challenge remains to develop bright probes to address specific biological processes in living cells or for cell tissue samples.[3,7,8] Recently, we have developed very bright europium triazacyclononane (TACN) complexes that stain various intracellular compartments, such as mitochondria, following cell uptake by macropinocytosis.[9–12] Such behavior is disadvantageous in the development of assays for membrane proteins, such as G protein-coupled receptors (GPCR).[13,14] With all established complexes,[9–12] the nonspecific interactions[3,15] caused by the probe adsorbing to the cell membrane or proteins within, cellular uptake, or the labeling of immature proteins within the cytosol, reached values that were not compatible with homogeneous time-resolved fluorescence (HTRF) bioassays.[3] Herein, we describe the synthesis of europium TACN complexes and their bioconjugates with improved water solubility that are incapable of entering or adhering to cells at concentrations less than 1 μM. The bioconjugates were evaluated in time-resolved Förster resonance energy transfer (TR-FRET) ligand-binding assays on living cells expressing the cholecystokinin-2 (CCK2) receptor both in plate-based format as well as using TR-FRET microscopy.

As the cell membrane consists of phospholipids and is considered to be negatively charged, we developed TACN complexes which are highly water soluble and negatively charged at physiological pH (Scheme 1). We reasoned that this should decrease interactions with the cell membrane as a result of repulsive Coulombic interactions. Moreover, the hydrophilic nature of the sulfonate and carboxylate groups should mask the inherent hydrophobic nature of the three aryl–alkynyl groups. The emission spectra and photophysical properties of the parent complexes, for example [EuL^1] and ring-substituted analogues such as [EuL^2][16], do not change significantly, indicating that the local C_3 symmetry around the Eu^{3+} ion is not compromised. Thus, bioconjugation of the



Scheme 1. Structures of parent and carbon-substituted Eu complexes [EuL^1], [EuL^2], [EuL^3], and [EuL^4].

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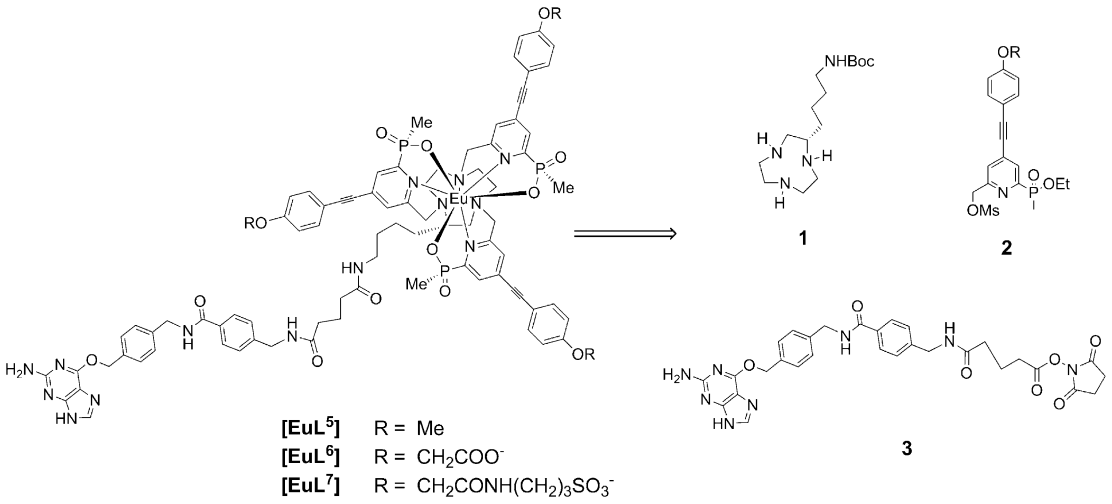

Scheme 2. Retrosynthesis of the target Eu conjugates [EuL^5], [EuL^6], and [EuL^7].

complexes was initiated by using derivatives substituted at the ring carbon atom of the TACN macrocycle, for example, those based on [EuL^3] (Scheme 1). This approach avoids varying the nitrogen substituents on the macrocyclic ring, as asymmetry in the aryl–alkynyl antennae complicates the synthesis.[9]

The synthesis of complexes [EuL^5], [EuL^6], and [EuL^7] was undertaken in a modular manner, using three key intermediates (Scheme 2). The carbon-substituted TACN intermediate, **1**, was prepared by an adaptation of an established method (Supporting Information),[16] wherein copper binding to the three ring N atoms allows the remote primary amine to react selectively with Boc_2O (Boc = *tert*-butoxycarbonyl). Copper is rapidly removed from the reaction mixture by bubbling H_2S through the solution, and the three N atoms on the macrocycle were alkylated with pyridyl mesylate derivative **2** (Scheme 2).[9,11] The benzylguanine moiety was introduced following reaction of the corresponding *N*-hydroxysuccinimide (NHS) ester **3** with the primary amine substituent on the ring that had been unmasked by deprotection of the BOC precursor (the deprotection was achieved using trifluoroacetic acid at room temperature).

The water solubility of these and related complexes was compared by assessing the partition coefficient (log *P*) of the complexes in water/octanol mixtures. Three equimolar solutions of complex were prepared in MeOH. The solvent was removed under reduced pressure and the resulting solid was dissolved and stirred for 24 hours in a mixture of water/octanol (0.9 mL; ratios 2:1, 1:1, or 1:2) giving a total concentration of approximately 2 μM. After equilibration, an emission spectrum for each layer was recorded in MeOH (50 μL of solution in 1 mL of MeOH). For each mixture, the log *P* value was calculated (see Table 1 for values; further details provided in the Supporting Information).

The introduction of sulfonate or carboxylate groups onto the ligand enhances the water solubility of complexes [EuL^{4a}], [EuL^{4b}], and [EuL^{4c}] significantly, and decreases the log *P* value from +1.4 to −2.2 (Table 1). The absorption and emission spectrum (shown in Figure 1 for complex [EuL^{4c}]) and the excited-state lifetimes hardly change when compared to the parent compounds.[11] However, the overall luminescence quantum yield in water drops slightly, suggesting that the energy transfer from the ligand intramolecular charge-transfer (ICT) excited state to Eu^{3+} is less efficient in water than in methanol.[9]

Table 1: Photophysical properties and log *P* values of the [EuL^4] complexes (295 K, H_2O).

Complex	λ_{max} [nm]	τ_0 [ms]	Φ [%]	ε [mM^{-1} cm^{-1}]	log *P*
[EuL^{4a}]	330	1.03	24	58	+1.4
[EuL^{4b}]	330	1.04	28	58	−2.2
[EuL^{4c}]	330	1.01	26	58	−2.2

After solving the problem of water solubility, we evaluated the complexes using SNAP-tag technology[17–21] on the cholecystokinin-2 (CCK2) receptor, a G protein-coupled receptor which has elevated expression levels when transiently transfected in HEK293 cells. The labeling of the benzylguanine derivatives [EuL^5], [EuL^6], and [EuL^7] on living HEK293 cells or HEK293 cells expressing the SNAP-tagged CCK2 (SNAP-CCK2)[21] was measured by monitoring the time-gated luminescence intensity at $\lambda = 620$ nm (Figure 2b–d).

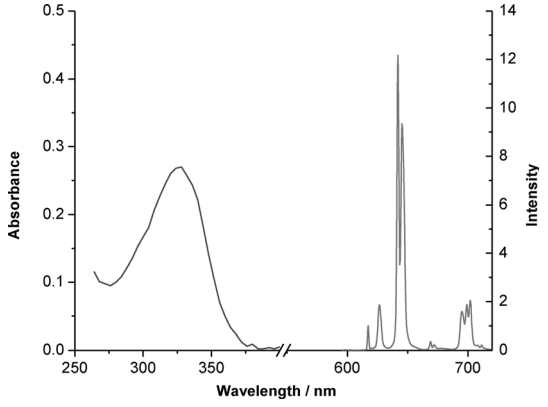

Figure 1. UV/Vis absorption and emission spectra for [EuL^{4c}] (295 K, water).

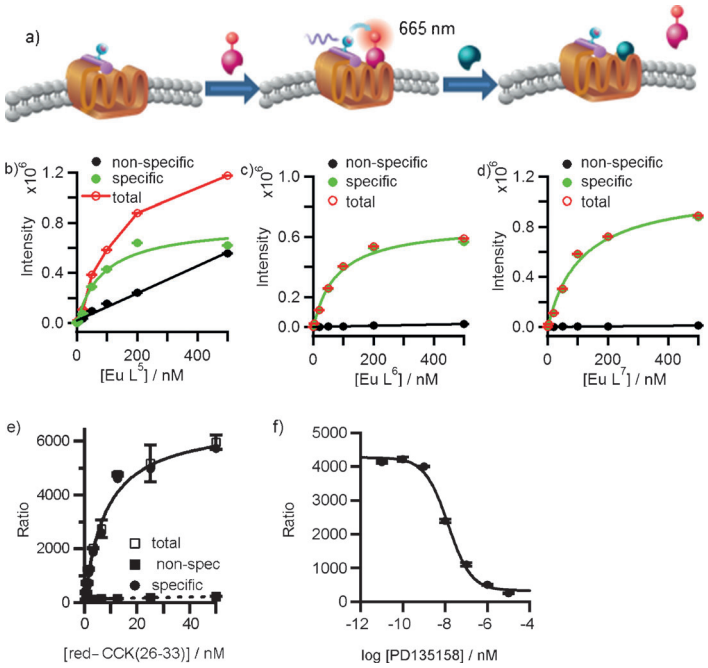

Figure 2. a) Representation of a TR-FRET ligand-binding assay on HEK293 cells expressing SNAP-CCK2,[3] showing the binding of red-CCK(26-33) (red sphere) to the SNAP-tagged CCK2 membrane receptor (orange). The TR-FRET signal is annihilated following the competitive binding of the natural CCK(26-33) agonist (blue sphere). b), c), d) Saturation curves for labeling HEK293 cells expressing SNAP-CCK2 and non-transfected HEK293 cells at the cell surface monitoring the time-gated luminescence intensity ($\lambda = 620 \pm 5$ nm, 60–460 μs) with complexes [EuL^5] (b), [EuL^6] (c), and [EuL^7] (d). e) Binding of red-CCK(26-33) to HEK293 cells expressing SNAP-CCK2, labeled with [EuL^7] (200 nM), monitored by following the ratio of the emission intensity at wavelengths $\lambda = 665$ and 620 nm ×10000. The nonspecific binding signal is measured by adding the unlabeled CCK(26-33) compound (10 μM). f) TR-FRET competition binding assay, monitoring the dose–response curve for the displacement of red-CCK(26-33) by CCK2 antagonist PD135158. The ratio of the emission intensity at wavelengths $\lambda = 665$ and 620 nm ×10000 was measured.

Significant labeling of non-transfected HEK293 cells occurs with the less hydrophilic compound [EuL^5], whereas with [EuL^6] and [EuL^7] this nonspecific labeling is negligible. These excellent results prompted us to perform a TR-FRET ligand-binding assay using a fluorescent agonist of the CCK2 receptor.

Addition of increasing concentrations of the red-fluorescent agonist of CCK2, red-CCK(26-33),[21] to [EuL^7]-labeled living SNAP-CCK2 cells and measuring the time-resolved FRET at $\lambda = 665$ nm gave rise to a saturating binding curve (Figure 2e). Note that upon addition of 10 μM of the natural agonist CCK(26-33) at the different concentrations of fluorescent ligand, the TR-FRET signal is annihilated because of the competition between the red-luminescent and the non-labeled compounds. By applying a one-site binding model to the specific binding data, a dissociation constant (K_d) of the red agonist of 8 nM was calculated, similar to previously reported data.[21] A TR-FRET competition binding assay shows that binding of 10 nM of red-CCK(26-33) is completely reversed in a dose-dependent way by adding excess quantities of the antagonist PD135158 (Figure 2f). The inhibition constant (K_i) is calculated to be 6 nM, in agreement with previously reported values.[21]

As the nonspecific labeling was suppressed, we could also monitor the labeling of HEK293 cells expressing the SNAP-tagged CCK2 with [EuL^7] using time-resolved microscopy. HEK293 cells (50k) were plated on a Lab-Tek slide, transfected with SNAP-CCK2 plasmid, and incubated for 2 days, after which time they were labeled with [EuL^7] (200 nM) for 1 hour. Using a delay of 100 μs and a gate time of 2 ms, a typical time-resolved image recorded with a filter of $\lambda = 615 \pm 10$ nm is shown in Figure 3a. Labeling clearly occurs at the cell surface and not within the cell.

It is only recently that lanthanide-based time-resolved FRET microscopy has been reported,[3,9,22,23] using primarily a Tb complex.[3,24,25] We tested whether this method can also be applied with these europium complexes to monitor ligand–receptor interactions on GPCR. As a bandpass filter at $\lambda = 615$ nm was used to detect the TR signal, we could also use the same procedure to monitor the time-resolved FRET signal at $\lambda = 670$ nm. Similar to the plate-based assay (Figure 2e), the red-fluorescent agonist of CCK2 was added to the cells labeled with [EuL^7]. The TR-FRET image, recorded at $\lambda = 670 \pm 20$ nm, suggests the formation of vesicles, indicating that many of the receptors previously expressed at the cell surface are now internalized together with the red-fluorescent agonist through a ligand-induced internalization process. The image data for both the europium and the TR-FRET channel for the conditions with or without the red-CCK(26-33) agonist (see Figure 3) reveals that the TR-FRET signal under the same imaging conditions is well above the intensity caused by donor bleed-through in the TR-

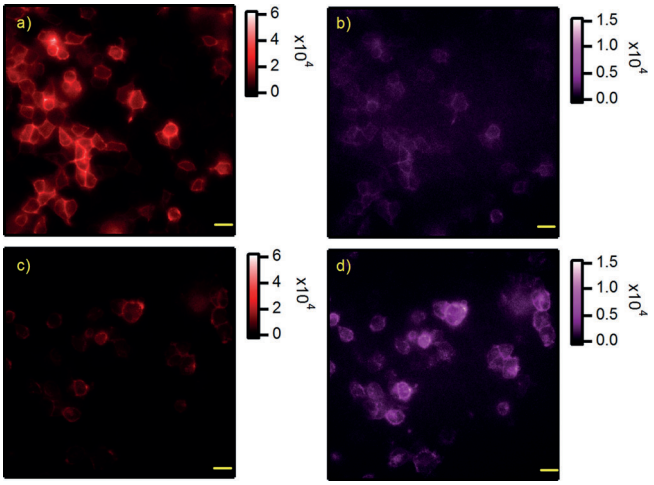

Figure 3. a) Time-resolved luminescence microscopy image of SNAP-CCK2-transfected HEK293 cells labeled with [EuL^7] (200 nM) monitored at $\lambda = 615 \pm 10$ nm ($\lambda_{ex} = 337$ nm, 30 Hz, $\tau_{delay} = 100$ μs, $\tau_{gate} = 2000$ μs). b) Bleed-through signal of the labeled cells in the TR-FRET channel ($\lambda = 670 \pm 20$ nm). c) Eu channel image, and d) TR-FRET channel image after adding red-CCK(26-33) to cells prepared and monitored as detailed in (a), incubating for 20 minutes, and washing three times with Tag-lite buffer. Scale bar = 20 μm in all images.

FRET channel. Similarly the Eu channel intensity is decreased as a result of the FRET process.

In conclusion, we have developed bright, kinetically stable, highly water-soluble Eu^{3+} complexes whose emissive properties are unchanged following bioconjugation. The carbon-atom substitution of the TACN ring with an aminoalkyl group (derived from *S*-lysine) creates a useful synthon that is easily functionalized with different antennae ligands[26] for use in a variety of applications. By introducing sulfonate or carboxylate groups onto the aryl–alkynyl antennae, nonspecific labeling of living HEK cells is suppressed, permitting the detection of GPCR-CCK2 using derivative [EuL^7]. Using time-resolved detection, the TR-FRET ligand-binding assays both on a plate reader and with TR-FRET microscopy are possible. The low nonspecific interactions of these complexes with cells will be a great advantage for the development of novel HTRF assays where specific biological interactions are studied. Furthermore, by introducing different electron-donating moieties on the aryl ring, we are developing bioconjugates whose electronic absorption bands are bathochromically shifted,[9,27] thereby improving their brightness for use in time-resolved confocal or two-photon microscopy.

Materials and Methods

Reagents: The Tag-lite labeling medium (LABMED), the SNAP-CCK2 plasmid for transient transfection of CCK2 receptors (PSNAPCCK2), and the red-fluorescent red-CCK(26-33) agonist (L0013RED) were obtained from Cisbio Bioassays. The CCK2 receptor antagonist PD135158 was purchased from Tocris. CKK(26-33) was obtained from Almac (Craigavon, UK). The 96-well plates were purchased from Greiner Bio-One (ref. 655086, Monroe, NC).

Cell culture: HEK293 wild-type cells were cultured in Dulbecco's modified Eagle's medium (DMEM) glutaMAX (1966-021; Invitrogen, Carlsbad, CA) supplemented with fetal bovine serum (10%), nonessential amino acids (1%), penicillin/streptomycin (1%), and HEPES (2 mM; HEPES = 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid).

Transfection procedure: Transient transfection was performed in 96-well plates using 100000 cells per well according to a previously described procedure.[21] Prior to cell seeding, the wells of the plates were precoated with poly-L-ornithine (50 μL) for 30 minutes at 37 °C. The transfection mixture (per individual well) was prepared by adding the SNAP-tag-CCK2 plasmid (100 ng) to optiMEM medium (49 μL), and Lipofectamine 2000 (0.8 μL; Invitrogen) and incubating for 20 minutes at room temperature prior to the addition to the plates. Subsequently, HEK293 cells (100 μL) at a density of 10^6 cell mL^{-1} was distributed in each well of the plates. The plates were incubated overnight at 37 °C under 5% CO_2.

Receptor labeling: For the labeling of SNAP-CCK2-expressed HEK293 cells with [EuL^5], [EuL^6], and [EuL^7], a previously described procedure[21] was used. A concentration series ranging from 0–500 nM was prepared in Tag-lite labeling medium. After incubation, the transfection mixture was removed from 96-well plates, and cells were treated with the prepared solutions (50 μL) and incubated for 1 hour at 37 °C under 5% CO_2. The residual compounds were removed by washing each well 4 times with Tag-lite labeling medium (100 μL).

Fluorescent ligand-binding assay: The affinity of red-CCK(26-33) for the CCK2 receptors was determined by incubating labeled cells with increasing concentrations of the fluorescent ligand. The nonspecific signal for each ligand concentration was determined by adding an excess of the corresponding unlabeled CCK(26-33) compound (10 μM). The unlabeled CCK(26-33) compound (20 μL) was added to plates containing labeled cells with Tag-lite labeling medium (100 μL), followed by the addition of the fluorescent red-CCK(26-33) agonist (20 μL). Plates were incubated at room temperature for 2 hours before signal detection.

Competitive binding assay: In competitive binding experiments, a fixed concentration of the fluorescent red-CCK(26-33) agonist (10 nM) was used in the presence of increasing concentrations of antagonist PD135158. PD135158 (20 μL) was added to plates containing labeled cells with Tag-lite labeling medium (100 μL), followed by the addition of the fluorescent red-CCK(26-33) agonist (10 μL). Plates were incubated at room temperature for 4 hours before signal detection.

Signal detection and data analysis: Signal detection was performed on PHERAstar FS plate reader (BMG LABTECH, Champigny-sur-Marne, France) at $\lambda = 620$ nm and $\lambda = 665$ nm (in TR mode: delay = 60 μs; time gate = 400 μs) upon $\lambda = 337$ nm laser excitation. Recorded data were analyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). Specific binding was determined by subtracting the nonspecific signal from the total signal. K_d values of the fluorescent ligand were obtained from the saturation curve of the specific binding. K_i values were calculated from competition assay experiments according to the Cheng and Prusoff equation.[28]

TR-FRET Microscopy: A Lab-Tek 8 chamber slide system was precoated with poly-L-ornithine (200 μL) and incubated for 30 minutes at 37 °C. The slide was washed with PBS (200 μL) and HEK293 cells were plated at a density of 5×10^4 cells per well (c/w) and incubated overnight at 37 °C under 5% CO_2. The following day, transfection was performed with optiMEM medium (100 μL), Lipofectamine 2000 (0.8 μL), and SNAP-CCK2 plasmid (1.2 μg per well). The Lab-Tek slide was further incubated for 2 days at 37 °C under 5% CO_2. After removal of the medium, SNAP-CCK2 receptors were labeled with [EuL^7] (200 nM) for 1 hour at 37 °C and washed twice with Tag-lite medium. The fluorescent red-CCK(26-33) ligand (10 nM) was added for the FRET signal detection. Hoechst 33342 (2 μg mL^{-1} per well) was added and after 20 minutes incubation at room temperature and washing twice with Tag-lite medium, the Lab-Tek slide was observed with a Zeiss oil immersion objective (40× magnification, 1.3 F-Fluar) on the Zeiss Axiovert 200 M TR-FRET inverted microscope equipped with a pulsed nitrogen laser (λ = 337 nm, 30 Hz) and a cooled intensified CCD camera PI Max 1024X1024 GenIII. For the luminescence imaging of [EuL^7], a $\lambda = 615 \pm 10$ nm bandpass filter was used with $\tau_{delay} = 100$ μs, τ_{gate} = 2000 μs, and at 60 gates per exposure. TR-FRET images were collected with a $\lambda = 670 \pm 20$ nm bandpass filter using the same procedure. The recorded data were analyzed using ImageJ software.

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