$\alpha_v\beta_3$-Integrin-Targeting Lanthanide Complex: Synthesis and Evaluation as a Tumor-Homing Luminescent Probe

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Abstract: The application of lanthanide complexes in the time-resolved fluorescence imaging of living cells has emerged in the last few decades, providing high-contrast images of cells through detection of the delayed emission. In the present study, we synthesized novel trivalent lanthanide complexes containing the cyclic peptide c(RGDfK) to visualize the $\alpha_v\beta_3$-integrin-expressing tumor cells. Conjugation of c(RGDfK) with the macrocyclic bipyridine ligand had little effect on the fluorescence properties of the complex, indicating that the coordinated lanthanide ion was well isolated from the peptide. Bright luminescence images of $\alpha_v\beta_3$-integrin-expressing U87-MG cells were successfully obtained by employing the probes.

Keywords: Cyclic RGDfK peptides; Time-resolved fluorescence imaging; U87-MG; Macrocyclic compound; Lanthanide complexes

Luminescence from trivalent lanthanide ions is characterized by narrow-band emission spectra, long lifetimes (of the order of milliseconds), and a large gap between the excitation and emission bands. Because of their long emission lifetimes, it is possible to carry out time-resolved detection of the complexes in order to eliminate background signals and autofluorescence from biomolecules.[1-6] Time-resolved fluoroimmunoassay has been well established as one of the applications of such lanthanide complexes.[7-8] For the improvement of the luminescence intensity of lanthanide complexes, it is desirable for the ligand structure to show intense absorption in the UV-Vis wavelength region, and to exhibit efficient energy transfer from the excited state of the ligand to the coordinated lanthanide ion. In addition, the ligand should form a strong complex with the lanthanide ion and shield the metal ion from nonradiative deactivation by solvent molecules.

In the present study, we synthesized trivalent lanthanide complexes of macrocyclic bipyridine ligands bearing cyclic peptides for the visualization of tumor cells expressing integrins on their surfaces. For the purposes of optical imaging of tumor cells and their functions, appropriate functionalization of the ligand structure is necessary, but such modifications often alter the luminescence properties. Integrins are transmembrane glycoproteins consisting of α and β subunits that mediate cell-to-cell and cell-to-extracellular matrix interactions.[9-12] In particular, the integrin $\alpha_v\beta_3$ appears to play an important role in tumor growth, metastasis, and angiogenesis, and thus the expression of this integrin is correlated with tumor aggressiveness. Cyclic peptides containing the RGD (Arg-Gly-Asp) sequence are well known as specific ligands of the $\alpha_v\beta_3$ integrin cell-surface receptor. The ability to visualize and quantify the $\alpha_v\beta_3$ integrin expression level will provide new opportunities for following integrin expression in tumor tissues.[13-17] As reported below, we introduced cyclic (Arg-Gly-Asp-*D*-Phe-Lys) [c(RGDfK)] into a lanthanide complex for molecular imaging of integrin-expressing tumor cells, and investigated the effects of the functionalization of the ligand structure on the luminescence properties.

(Insert Fig. 1)

The aims of the present study are to develop a novel $\alpha_v\beta_3$-integrin-targeting fluorescent probe, and to investigate the effects of side chains for the introduction of c(RGDfK)[18,19] peptides on its fluorescence properties. It has been demonstrated that bipyridine-cryptand ligands can enhance the fluorescence lifetimes of their lanthanide complexes by shielding the trivalent lanthanide ions from water molecules.[20-23] We designed a macrocyclic bipyridine ligand bearing two c(RGDfK) peptides as tumor-targeting moieties (**1**, Fig. 1). The macrocyclic compound **2** was synthesized from 6,6′-dimethyl-2,2′-bipyridine in three steps, following a previously reported procedure.[24,25] For the conjugation of the c(RGDfK) peptide, ligand **2** was derivatized to form the dicarboxylic acid **3**, and then reacted with *N*-(2-aminoethyl)maleimide to afford ligand **4** (Scheme 1). Coupling between the thiol-derivatized c(RGDfK) peptide [c(RGDfK)-SH] and **4** yielded **1** and the monosubstituted ligand **5**. Trivalent europium (Eu^{3+}) and terbium (Tb^{3+}) complexes of **1**, **3**, and **5** were prepared by adding lanthanide chlorides ($LnCl_3$) to solutions of the corresponding ligands.

(Insert Scheme 1)

(Insert Fig. 2)

Fig. 2 shows the UV absorption spectra of $Eu^{3+}\subset$**1**, **3** in H_2O. The absorption band at around 310 nm is mainly due to the bipyridine structure of the ligands. Fluorescence spectra of the complexes $Ln^{3+}\subset$**1** (Ln^{3+} = Eu^{3+} and Tb^{3+}) were obtained upon excitation at 315 nm, and showed characteristic luminescence spectra arising from the emissive $^5D_0$ level to the ground-state ^7F_J (J = 0–4) level of

Eu^{3+}, or from the $^5D_4$ level to the ^7F_j (j = 3–6) level of Tb^{3+} (Fig. 3). It has been well established that light-harvesting ligands absorb UV light and transfer energy from the excited states to the coordinated lanthanide ions.[26,27] In fact, other complexes ($Ln^{3+}\subset$**3**, **5**) showed quite similar fluorescence spectra (data not shown). In order to gain further understanding of the energy-transfer process in our complexes, we estimated the energy level of the excited triplet state of **3** from its phosphorescence spectrum obtained at 77 K (Supplementary Information, Fig. S1). Phosphorescence from ligand **3** showed a maximum at around 480–490 nm (20800–20400 cm^{-1}), and was efficiently quenched in the case of the corresponding Eu^{3+} complex ($Eu^{3+}\subset$**3**), which suggests that the emission process involves energy transfer from the excited triplet state of the ligand to Ln^{3+}.

(Insert Fig. 3)

(Insert Table 1)

The quantum yields (Φ_F) and lifetimes (τ) of the emission are listed in Table 1. Emission quantum yields obtained from the complexes are relatively low, because back electron transfer deactivation from the excited Ln^{3+} to the triplet energy levels of the ligands is likely to occur due to small band gaps between them. The hydration number (the number of water molecules, q) of the inner coordination sphere of the lanthanide ions can be estimated by measuring the differences between the emission lifetimes in H_2O and D_2O using the equations of Horrocks and Parker.[28-30] The fluorescence lifetimes of the complexes were determined to be ≈1 ms, which should be long enough for the time-resolved fluorescence imaging of biomolecules to discriminate the fluorescence of these complexes from autofluorescence originating from the tissue. The very slight shortening

of their lifetimes observed in phosphate buffer encouraged us to use the probes for the microscopic observation of biomolecules under physiological conditions. The *q*-values obtained for the Eu^{3+} and Tb^{3+} complexes are 0.2–0.8, which suggests that there is less than one molecule of water in the first coordination sphere. The fluorescence lifetime and *q*-value for $Eu^{3+}\subset$**2** were also obtained as 0.2 ms and 3.2 (in H_2O), respectively. Thus, the carbonyl groups on the linkers of $Ln^{3+}\subset$**3** should contribute to the coordination of Eu^{3+}. Considering that $Ln^{3+}\subset$**1** and $Ln^{3+}\subset$**3** showed similar *q*-values, the c(RGDfK) residues may not be directly coordinated to the central lanthanide ion. It is noteworthy that the lifetime of $Tb^{3+}\subset$**1** in H_2O (1.9 ms) is rather longer than those of other Tb^{3+} complexes (0.8–1.0 ms), probably because the c(RGDfK) peptides of $Tb^{3+}\subset$**1** prevent deactivation by the closely diffusing water molecules around Tb^{3+}.[29,30] Unlike the Eu^{3+} complexes, emission from the Tb^{3+} complexes (Φ_F = 0.17) is intense enough for cell imaging. This is partly because of smaller energy gap between $^5D_0$ and $^7F_6$ states of Eu^{3+} than that between $^5D_4$ and $^7F_0$ of Tb^{3+} enhances non-radiative deactivation of the excited states of Eu^{3+}.[8,19]

(Insert Fig. 4)

The cytotoxicity of complexes $Eu^{3+}\subset$**1** and $Tb^{3+}\subset$**1** was assayed by the WST-8 method (Fig. S2).[31] Both probes induced cell aggregation, but showed no remarkable toxicity in the concentration range below 100 μM (IC_{50} > 100 μM). The stability of $Eu^{3+}\subset$**1** and $Tb^{3+}\subset$**1** was evaluated during a period of 42 h at 37 ºC in the medium. By emission analysis it was found that both complexes were sufficiently stable enough for the microscopic observation (Fig. S3). Fluorescence imaging of living tumor cells was examined by incubating the probes with highly

$\alpha_v\beta_3$-integrin-expressing human glioblastoma U87-MG cells or with less expressing human prostate cancer PC-3 cells at 37°C. As shown in Fig. 4, the U87-MG cells were successfully visualized by $Eu^{3+}\subset$**1** upon excitation at 330 ± 40 nm and fluorescence collection at 605 ± 28 nm (Fig. 4c, d). On the other hand, only very weak fluorescence was observed from PC-3 cells incubated with $Eu^{3+}\subset$**1** (Fig. 4a, b) and U87-MG cells incubated with $Eu^{3+}\subset$**3** (Fig. 4e, f). These results clearly suggest that the lanthanide complex $Eu^{3+}\subset$**1** recognizes the integrin $\alpha_v\beta_3$ on the surface. The effect on microscopic observation of mono- and disubstitution of the ligand with the c(RGDfK) unit was also examined by incubating $Tb^{3+}\subset$**1** or $Tb^{3+}\subset$**5** with U87-MG cells under the same conditions (Fig. S4). It has been demonstrated that multimeric RGD peptides show enhanced binding affinities toward integrins as compared to monomeric peptides;[13] however, no remarkable differences were observed between the fluorescence microscopic images. This is partly because the two c(RGDfK) units are not sufficiently well separated to bind two neighboring $\alpha_v\beta_3$ integrin sites simultaneously.[13]

In summary, we have synthesized c(RGDfK)-linked macrocyclic bipyridine as ligands for trivalent lanthanide ions (Eu^{3+} and Tb^{3+}) in order to utilize them as time-resolved fluorescence probes for $\alpha_v\beta_3$-integrin-expressing tumor cells. It has been demonstrated that the introduction of c(RGDfK) units into the bipyridine macrocycle has little effect on the fluorescence properties, because the bipyridines and the linkers shield the central lanthanide ion from c(RGDfK) and solvent molecules. Successful visualization of the target tumor cells has encouraged us to develop novel time-resolved fluorescence probes that can be photoexcited by lower-energy UV light than that employed in this study.

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Captions

Fig. 1 Structure of c(RGDfK)–macrocyclic bipyridine ligand (**1**).

Fig. 2 UV absorption spectra of Eu^{3+}⊂**1** (solid line) and Eu^{3+}⊂**3** (dashed line); concentration 10 μM in H_2O.

Fig. 3 Steady-state emission spectra of Eu^{3+}⊂**1** (10 μM, solid line) and Tb^{3+}⊂**1** (0.5 μM, dashed line) in H_2O. Complexes were photoexcited at 315 nm (excitation and emission slit widths: 5 nm, respectively).

Fig. 4 Microscopic images of live PC-3 (a, b) and U87-MG (c–f) cells incubated with (a–d) Eu^{3+}⊂**1** (100 μM), and (e, f) Eu^{3+}⊂**3** (100 μM). (a, c, e) Phase-contrast images, and (b, d, f) merged images of fluorescence and phase-contrast pictures.

Scheme 1. Reagents and conditions: (a) NCS, BPO, CCl_4, reflux, 24 h, 25%; (b) TsNHNa, DMF, 80°C, 5 h; (c) conc. H_2SO_4, 110°C, 2 h, 30% in two steps; (d) Ethyl bromoacetate, K_2CO_3, CH_3CN/CH_2Cl_2, reflux, 24 h; 61%; (e) KOH, EtOH, r.t., 3 h, 70%; (f) *N*-(2-aminoethyl)maleimide, HCTU, Et_3N, CH_3CN, r.t., 6 h, 30%; (g) c(RGDfK)-$SHCOCH_3$, NH_2OH·HCl, EDTA-HEPES buffer, r.t., 4 h.

Table 1. Fluorescence lifetimes and quantum yields of the complexes. Fluorescence lifetimes were measured for complexes **1**, **3**, and **5** in H_2O, D_2O, and phosphate buffer saline (PBS).

Table 1.

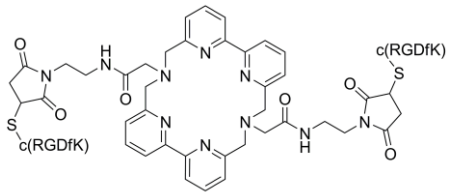
Ligand	**1**		**3**		**5**	
Ln^{3+}	Eu^{3+}	Tb^{3+}	Eu^{3+}	Tb^{3+}	Eu^{3+}	Tb^{3+}
τ_{H2O} (ms)	0.8	1.9	0.5	0.8	0.7	1.0
τ_{D2O} (ms)	1.6	2.5	1.0	1.0	1.1	1.3
τ_{PBS} (ms)	0.7	1.8	0.6	0.9	0.9	1.2
q [a,b]	0.4	0.5	0.8	0.8	0.2	0.8
Φ_F [c]	0.05	0.17	0.01	0.17	0.03	0.06

[a] $q(Eu) = 1.11 \times (1/\tau_{H2O} - 1/\tau_{D2O} - 0.31)$, ref. 29.

[b] $q(Tb) = 5.0 \times (1/\tau_{H2O} - 1/\tau_{D2O} - 0.06)$, ref. 30

[c] Measured in H_2O.

Figure 1.



1

Figure 2.

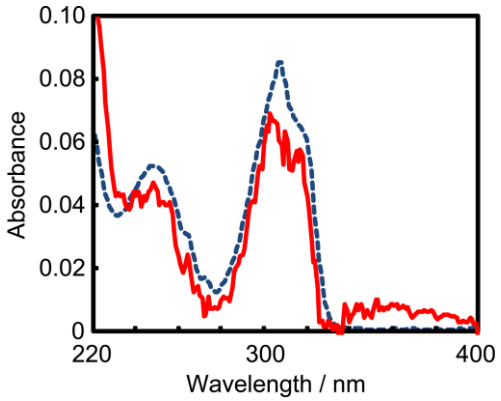


Figure 3.

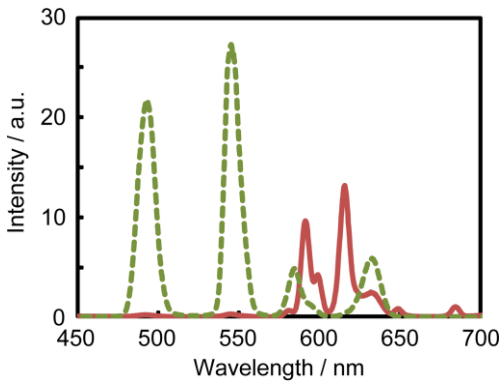
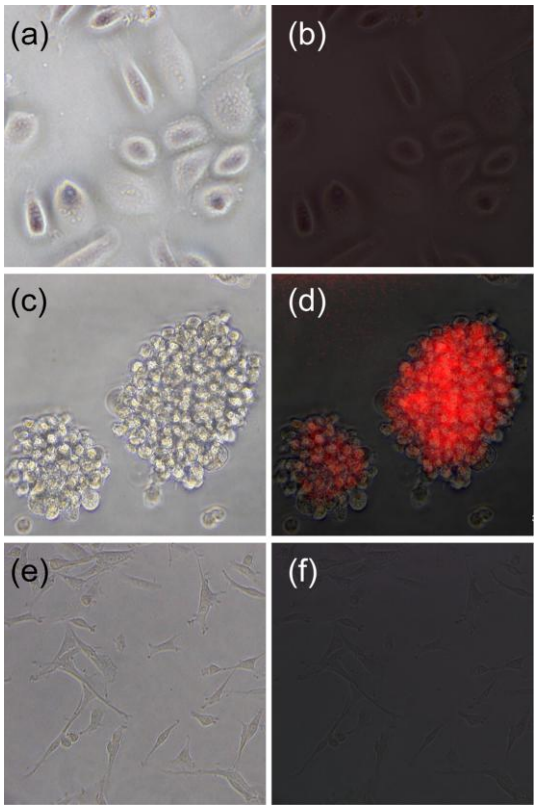
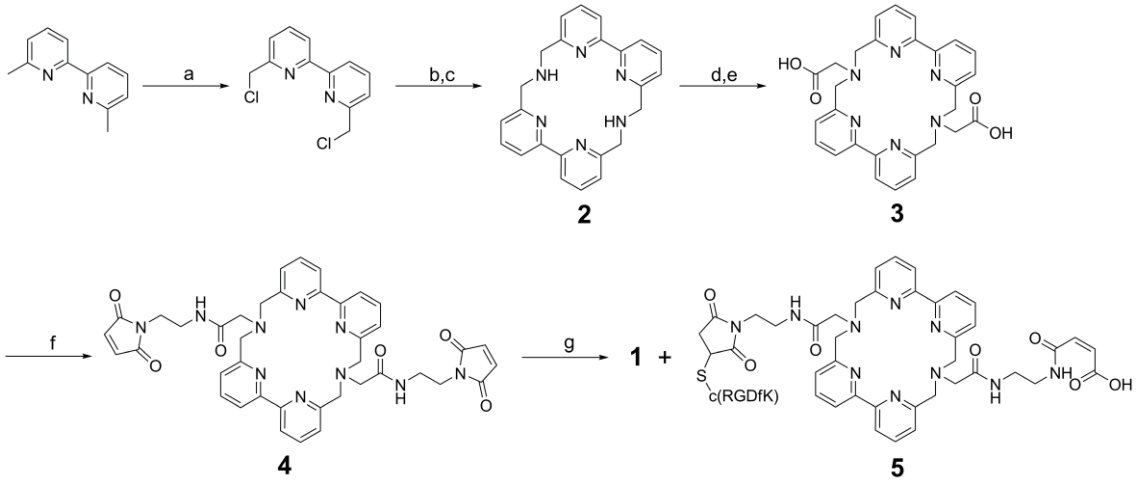


Figure 4.



Scheme 1



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