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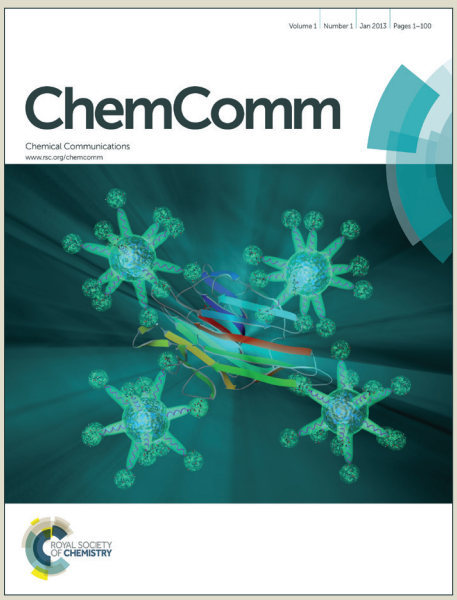

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Real-time in-situ monitoring via europium emission of the photo-release of antitumor cisplatin from a Eu-Pt complex

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A water-soluble light-responsive antitumor agent, PtEuL, based on a cisplatin-linked europium-cyclen complex has been synthesized and evaluated for controlled cisplatin release by linear/two-photon excitation *in vitro* with concomitant turn-on and long-lived europium emission as responsive traceable signal.

Cisplatin is a highly effective chemotherapeutic drug against a variety of solid tumors, such as testicular and non-small cell lung cancers. It exerts its anti-cancer activity mainly via an extensive DNA-adduct formation which triggers apoptotic cell death.[1] However, its vulnerability to attack by various proteins in blood, notably serum albumin and glutathione, which hampers its delivery to the disease targets, resulting in many severe side effects (e.g., leucopenia, nephrotoxicity) that has limited its further application.[2] To overcome this problem, many cisplatin analogues which slow down its reaction with protein thiols have been developed. Other strategies such as its controlled release via encapsulation by micelle, nanomaterials as well as photo-activated Pt(IV) prodrug (i.e., systemic transport of cisplatin in a relatively inactive form and then re-activate it by light at the target sites) have also been developed.[3]

In this proof-of-concept study, we propose to develop a water-soluble lanthanide-cisplatin complex, **PtEuL** (**Pt**: platinum(II); **Eu**: europium(III); **L :** 2,2',2''-(10-((4-((4-(isonicotinamido)phenyl)ethynyl) pyridin-2-yl)methyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)tri-acetic acid (Fig. 1)) containing the cisplatin which can be released only upon photo-irradiation. Concomitant to this photo-dissociation, the originally non-emissive **Eu** center of the **Pt-Eu-L** complex now became highly emissive, thus allowing a direct monitoring of the photorelease of the cytotoxic cisplatin. Such off-on responsive europium emission can confirm the on-target delivery and release of cisplatin *in vitro*. The design of our **PtEuL** complex is based on the unique photophysical properties of the lanthanide ions.[4] Owing to their forbidden f-f radiative transitions, appropriate organic antenna chromophores are required to absorb and transfer the photo-excitation energy to the lanthanide ions, thus amplifying their inherently weak but hypersensitive fingerprint emissions.[5] Our water-soluble and cell-permeable **PtEuL** complex is constructed from a Eu(III)-cyclen complex containing a rigid π-conjugated antenna chromophore with an isonicotinamide ligand which is coordinated to the cisplatin (Fig. 1). Due to the close proximity to the Pt center, the excitation energy absorbed by the antenna chromophore is channeled mostly via intersystem charge transfer to the dissociative states of Pt and no Eu emission is detected. Upon photo-dissociation of the cisplatin from **PtEuL**, energy transfer from the antenna's triplet excited state to the first excited state of Eu(III) takes place with significantly enhanced Eu emission in an off-on manner. This design complex provides a real-time traceable delivery vehicle for cisplatin to its *in vitro* target. Comprehensive photophysical and *in vitro* studies of this complex, such as measurements of its quantum yield, sensitization efficiency, emission lifetime, DNA binding and cleavage properties, dark cytotoxicity and photocytotoxicity, have been conducted. The results obtained support the application of **PtEuL** as a traceable and photo-activatable prodrug for the real-time optical monitoring of the controlled and targeted delivery of cisplatin.

In this study, cisplatin-free Eu complex **EuL** was prepared as the control for comparison. In addition, two gadolinium analogues, **GdL** and **PtGdL**, were synthesized for probing the triplet state of the cyclen-based ligand **L**. All the complexes, **PtLnL** and **LnL** (**Ln** = **Eu** and **Gd**) were obtained using synthetic scheme S1 and characterized by HRMS and HPLC.

The solution-state electronic ansorption and emission spectra of all the complexes and ligand were recorded at 298 K. (Fig. 2) The EuL complex is the proposed product of PtEuL after photo-

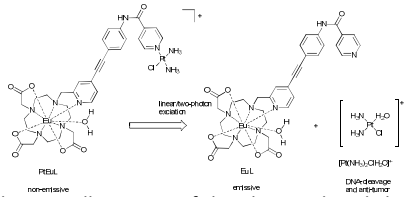

Fig. 1 The schematic illustration of the photo-induced cleavage of our photo-responsive anticancer bioprobe **PtEuL** to generate highly luminescent europium off-on signals and cytotoxic effects.

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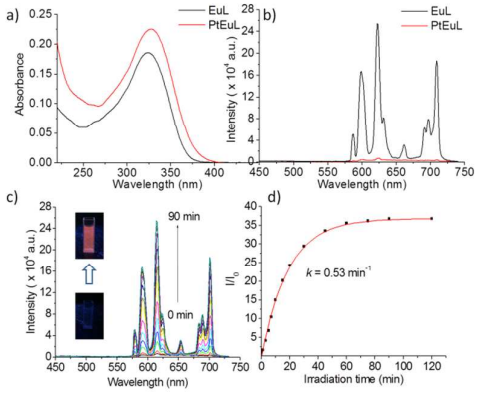

Fig. 2 (a) Absorption and (b) emission spectra of **PtEuL** and **EuL** (3 μM, λ_{ex} = 325 nm) in aqueous solution. c) Emission spectral variation of **PtEuL** under UVA (365 nm) irradiation, light dosage = 4 J cm^{-2} for 90 min. (c insert) Photographic image of europium emission enhancement of **PtEuL** under UVA irradiation for 20 min; d) plot of I/I_0 @ 615nm vs. time. Pseudo-first order rate constant k = 0.53 min^{-1}.

dissociation and was used to monitor the photo-dissociation of **[Pt-(NH$_3$)$_2$Cl]$^+$** from **PtEuL**. The absorption bands of **PtEuL** and **EuL** are similarly located at *ca*. 327 and 324 nm, respectively, but the absorption coefficient of **PtEuL** is 22600 M^{-1} cm^{-1}, which is 4000 M^{-1} cm^{-1} higher than that of **EuL**. (Fig. 2a) However, the emission quantum yields of **PtEuL** and **EuL** show an opposite trend to their absorption coefficients. **EuL** exhibited strong red emission, with 11% quantum yield, in aqueous solution, while **PtEuL** gave only very weak Eu emission under identical experimental conditions. (Table 1 and Fig. 2b) The room temperature emission spectra of **PtEuL** and **EuL**, obtained under UV excitation (λ_{ex} = 325 nm) of the antenna ligand are given in Fig. 2b, which show the characteristic $^5D_0 \rightarrow {}^7F_J$, where J = 0 - 4 transitions of Eu^{3+}. The ratios of the $^5D_0 \rightarrow {}^7F_J$ emissions, where J = 0 - 4, from **PtEuL** and **EuL** indicated that both complexes have similar coordination geometry. (Fig. 2b) The water soluble complex **EuL** showed intensive sensitized Eu emission because the energy level of the T_1 excited state of the antenna chromophore is around 20202 cm^{-1}, which falls in the optimum energy transfer gap to the first excited state of Eu(III), $^5D_0$ (17300 cm^{-1}). (6.17 μs, Fig. S7) But the phosphorescence spectra of **PtGdL** obtained at 77 K shows the phosphorescence band of the antenna ligand at 445 nm (22222 cm^{-1}, 6.51 μs, Fig. S7). This observation indicates that it is also possible to transfer energy to the $^5D_1$ excited state of Eu(III), populating it first and then undergoes fast nonradiative energy transfer to the $^5D_0$ state, which then emits. However, in the Pt-Eu complex, **PtEuL**, only limited energy transfer from the triplet excited state of the antenna ligand to the Eu excited states occurs, resulting in insignificant Eu emission.[6] This observation can be explained by the presence of dissociative states on Pt(II) proposed by Sadler et al. in their study of one- and two-photon induced dissociation of rigid π-conjugated ligand from a Pt(II) complex,[7] which is structurally similar to our complex. The proposed energy transfer mechanism of **PtEuL** is summarized in Fig. S8. In **PtEuL**, the energy absorbed by the antenna ligand is transferred to the dissociative states of the *cis*-Pt(II) moiety and therefore very limited energy can be transferred to the Eu(III) excited state for emission. This unusual photophysical property offers a responsive signal change upon UVA or two-photon excitation of the **PtEuL** complex, resulting in the dissociation of **[Pt(NH$_3$)$_2$Cl]$^+$** from the complex for covalent attack of DNA bases in the conventional cisplatin anticancer chemotherapy. The sensitized charge transfer first weakens the Py-Pt bond, which subsequently results in the dissociation of the cisplatin moiety. The Eu emission can now be observed in aqueous solution (Fig. 2c) for two-photon induced *in vitro* imaging. (Fig. 4, UV excitation is phototoxic) The photo-dissociation of **PtEuL** (3 μM in Tris-buffer, pH = 7.4, 50 mM NaCl) was monitored by the variation of the Eu emission (λ_{em} = 615 nm, $^5D_0 \rightarrow {}^7F_2$) with the irradiation time. The Eu emission intensity was enhanced by more than 35-fold (emission quantum yield increased by > 100-fold) after continuous excitation of **PtEuL** with UVA. (λ = 365 nm) (Fig. 2c) The pseudo-first order rate constant k was determined to be 0.53 min^{-1} under this experiment condition. (Fig. 2d) The photo-dissociation product of **PtEuL** after 90 min of UVA irradiation, was identified by HPLC analysis where the retention time and spectrum of the separated photoproduct was found to be in good agreement with those of the **EuL** synthesized (Fig. S9). As the major toxic effect of cisplatin and its analogs is mediated through its interaction with the free thiols present on proteins, its controlled release from the prodrug can enhance the interaction of the active cisplatin with its intended target at the delivered site.

Direct binding of **PtEuL** with DNA in dark were studied by circular dichroism (CD). The CD spectra of DNA in the presence (incubation time = 12 h) and absence of **PtEuL** are shown in Fig. 3a. The dramatic decrease in ellipticity for both the positive and negative bands of DNA in the presence of **PtEuL** indicates that **PtEuL** can directly bind to DNA and unwind its helix, leading to the loss of helicity.[10] (Fig. 3a) The interaction of photo-activated **PtEuL** with DNA was examined by agarose gel electrophoresis. (Fig. 3b) After UVA irradiation with **PtEuL**, conversion of the supercoiled conformer of the plasmid DNA to its open-circular conformer,

Table 1. Photophysical parameters and dark cytotoxicities of the Eu(III) complexes.

Complex	λ_{max}/nm [a]	ε/mol/L cm^{-1}[a]	E_{T1}/ cm^{-1}[b]	τ(H_2O)/ ms[c]	τ(D_2O)/ ms[c]	q ±0.2[d]	Φ_L^{Eu} /% [e]	IC_{50}/μM HeLa[h]	IC_{50}/μM A549[h]
EuL	324	18600	20202	0.62	1.99	1.0	11.0	> 500 [h]	> 500 [h]
PtEuL	327	22600	22222	-	-	-	< 0.1%	22.5±0.5	49.5±2.0

[a] Absorption coefficient in H_2O, 298 K; [b] Triplet energy level of chromophore obtained from the phosphorescence of the **PtGdL** complex (in H_2O/glycerol v:v = 1:1, 77 K). [c] Eu emission decay (λ_{em} = 615 nm, $^5D_0 \rightarrow {}^7F_2$, λ_{ex} = 325 nm); [d] Hydration number of Eu (III) complexes, q = 1.2 ×[k(H_2O) – k(D_2O) - 0.25], k = τ^{-1}; [8] [e] Overall Eu emission quantum yield in H_2O, determined by integrated sphere; [9] [f] Dark cytotoxicity of the complex on HeLa and A549 cells. Cisplatin is used as the control in this experiment and its IC_{50} on HeLa and A549 is 3.30 ± 0.1 μM and 8.70 ± 0.5 μM, respectively. IC_{50} > 500 μM is from the curve of the toxicity via sample concentration. IC_{50} is presented as the mean (μM) ± standard deviation of three independent experiments.

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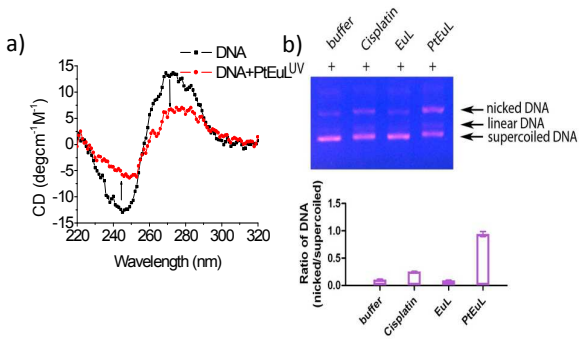

Fig. 3 a) CD spectra of plasmid DNA (20 nM in Tris-HCl buffer, pH = 7.4) treated with or without **PtEuL** (50 μM) in dark at 37°C for 12 h. b) Plasmid DNA was incubated with the test compounds (20 μM) as indicated, irradiated by UVA (light dose 50 J/m^2), and then subjected to agarose gel electrophoresis. The DNA gel images were obtained using GelRed Nucleic Acid Stain (BIOTIUM).

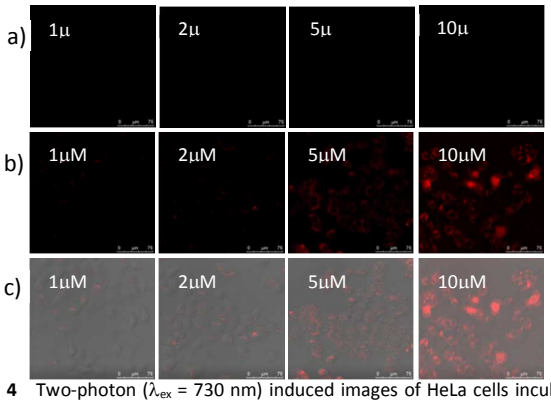

Fig. 4 Two-photon (λ_{ex} = 730 nm) induced images of HeLa cells incubated with **PtEuL** at different dose concentrations (0, 1, 2, 5, 10 μM) for 24 h. a) without irradiation, b) after 30 min excitation, c) merged images of (a) and (b).

which indicates DNA nicking (single strand break) and linear conformer, which indicates double strand break), is clearly seen. Interestingly, strong DNA photocleavage activity is only seen with **PtEuL**, but less significantly in cisplatin and not at all in **EuL**, suggesting that a DNA damage mechanism distinct from that of cisplatin is operating in **PtEuL**. The dark cytotoxicity of **PtEuL** was evaluated in two cancer cell lines (HeLa and A549), together with cisplatin. (Table 1. and Fig. S10) **PtEuL** is seen to exhibit a lower dark cytotoxicity, compared to cisplatin, towards the HeLa and A549 cells, with the respective IC_{50} of 22.5 ± 0.5 μM and 49.5 ± 0.1 μM. (Table 1) However, **EuL** showed no toxicity towards these two cancer cell lines under identical conditions.

Near infrared (NIR) excitation has increasingly been used in live cell imaging and photodynamic or photoactivated chemotherapy because NIR photon (λ = 650-900 nm) can penetrate deeper into tissues with pinpoint accuracy and little collateral photodamage.[10] The two-photon photophysical properties of some antenna ligands of lanthanide complexes have been studied previously.[12] The two-photon induced cytotoxicity of the **EuL** and **PtEuL** complexes towards HeLa cell after incubation at different dose concentrations (**PtEuL**: 0, 1, 2, 5, 10 μM; **EuL**: 0, 10, 20, 50, 100 μM) for 24 hours was studied by two-photon (λ_{ex} = 730 nm) confocal microscopy. (Fig. 4 and Fig. S11) Red emission from **PtEuL** was observed in this cell at > 2 μM after 30 min of intermittent two-photon excitation (10 s of laser excitation per min). This emission, which increased with **PtEuL** dose, is closely associated with the cell nuclei and nuclear membrane, which are the known intracellular targets of cisplatin, is presumably derived from the dissociated **PtEuL**. In contrast, no red emission was detected from the cells treated with **EuL** under identical conditions. This is probably due to the lower cellular uptake rate of **EuL** because we have observed no Eu emission from the HeLa cells even after incubation in 100 μM of **EuL**, ceteris paribus. (Fig. S11). The apparent slow dissociation rate of cisplatin from **PtEuL** without photo-activation (Fig. S10) is crucial to maintaining the stability of the prodrug before reaching its intended target. However, the release of cisplatin from the **PtEuL** prodrug only via photo-activation also limits its *in vivo* application to superficial malignancy, such as skin cancer, oral cancer and prostate cancer, due to the limited depth penetration of even NIR light through tissues.

In conclusion, we have designed and synthesized a platinum-europium complex (**PtEuL**) which holds great promise as a controlled delivery vehicle of cisplatin. In addition, the Eu emission produced upon the photo-dissociation of cisplatin from **PtEuL** allows a real-time monitoring of the cisplatin release *in vitro*, thus making it a luminescent imaging as well as antitumor chemotherapeutic agent.

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