Supplementary Material (ESI) for Chemical Communications
This journal is (c) The Royal Society of Chemistry 2012

Supporting Information

A luminescent tetranuclear ruthenium(II) complex as a tracking non-viral gene vector

Bole Yu, Yu Chen, Cheng Ouyang, Huaiyi Huang, Liang-Nian Ji and Hui Chao*

MOE Key Laboratory of Bioinorganic and Synthetic Chemistry, State Key Laboratory of Optoelectronic Materials and Technologies, School of Chemistry and Chemical Engineering, Sun Yat-Sen University, Guangzhou 510275, P. R. China. E-mail: ceschh@mail.sysu.edu.cn; Fax: 86-20-84112245; Tel: 86-20-84110613

Experimental Procedures

Instruments

Microanalysis (C, H, and N) was carried out with a Perkin-Elmer 240Q elemental analyzer. ^{1}H NMR spectra were recorded on a Varian-500 spectrometer at 25 ^{o}C. All chemical shifts are given relative to tetramethylsilane (TMS). Electrospray mass spectra (ES-MS) were recorded on a LCQ system (Finnigan MAT, USA). UV–Vis spectra were recorded on a Perkin-Elmer Lambda 850 spectrophotometer. Emission spectra were recorded on a PerkineElmer LS 55 spectrofluorophotometer at room temperature. Time-resolved emission measurements were conducted on an FLS 920 combined fluorescence lifetime and steady state spectrometer. Quantum yields of luminescence at room temperature were calculated according to literature procedures[1], compared to $[Ru(bpy)_3]^{2+}$ (Φ = 0.028 in aerated aqueous solution).[2]

Materials

EtBr, DAPI, DOPE, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Calf thymus DNA (CT-DNA) were purchased from Sigma. The plasmid pBR322 DNA from MBI Fermentas, the plasmid pEGFP DNA was from Clonetech, and the plasmid pGL3 control vector and luciferase kid were from Promega. Unless otherwise stated, the DNA concentration was expressed in base pairs. All samples were prepared using distilled water that had been passed through a Millipore-Q ultra-purification system.

Syntheses

The compounds $[Ru(bpy)_3]Cl_2{\cdot}2H_2O$[3], $Ru(DMSO)_4Cl_2$[4] and $Ru(bpy)_2(H_2bpib)Cl_2{\cdot}2H_2O$[5] were

synthesized according to literature methods.

***Synthesis of [Ru{(bpy)$_2$Ru(H$_2$bpib)}$_3$]Cl$_8$* (Ru1)**

This complex was synthesized according to the previously reported method[5] with some modifications. A mixture of Ru(bpy)$_2$(H$_2$bpib)Cl$_2$·2H$_2$O (0.156 g, 0.15 mmol), Ru(DMSO)$_4$Cl$_2$ (0.024 g, 0.05 mmol), and dimethyl formamide (10 mL) was refluxed under argon overnight. After the solvent evaporated under reduced pressure, the product was purified by SP-Sephadex C-25 cation exchange chromatography using a solution 0.5 M NaCl in water–acetone (5:3, V/V) as eluent. Yield 0.117 g, 73 %. (Found: C 57.81, H 3.72, N 15.37%. C$_{156}$H$_{102}$N$_{36}$Cl$_8$Ru$_4$·3H$_2$O requires C 58.14, H 3.38, N 15.65%). ^{1}H NMR (400 MHz, d$_6$-DMSO): δ 14.6 (s, 6H), 9.18 (br, 12H), 8.89 (d, J = 8 Hz, 6H), 8.85 (d, J = 8.4 Hz, 6H), 8.64 (s, 12H), 8.23 (t, J_1 = J_2 = 8 Hz, 6H), 8.12 (m, 18H), 7.97 (br, 12H), 7.87 (d, J = 5.2 Hz, 6H), 7.62 (d, J = 5.6 Hz, 6H), 7.60 (t, J = 6.8 Hz, 6H), 7.36 (t, J = 6.4 Hz, 6H). ES-MS (CH$_3$OH): m/z 960.0 ([M - 8Cl - 5H]$^{3+}$), 720.7 ([M - 8Cl - 4H]$^{4+}$), 576.5 ([M - 8Cl - 3H]$^{5+}$), 480.9 ([M - 8Cl - 2H]$^{6+}$), 412.4 ([M - 8Cl - H]$^{7+}$).

DNA Binding assay.

The DNA-binding experiment was performed at room temperature. UV-Vis spectra were recorded on a Perkin-Elmer Lambda 850 spectrophotometer and the spectroscopic titration was carried out in buffer A (5 mM Tris-HCl, 50 mM NaCl, pH 7.2).The DNA concentration per nucleotide was determined by absorption spectroscopy using the molar absorption coefficient (6600 M^{-1}cm^{-1}) at 260 nm[6]. A solution of CT-DNA in the buffer gave a ratio of UV absorbance at 260 and 280 nm of 1.8–1.9:1, indicating that the DNA was sufficiently free of protein[7].

Absorption titration experiments were performed by maintaining **Ru1** concentration (4 μM) and varying nucleotide concentration (0-40 μM) in buffer. The **Ru1**–DNA solutions were allowed to incubate for 5 min before the absorption spectra were recorded. The intrinsic binding constants K_b to DNA were determined using the eqn. 1[8]

$$[DNA]/(\varepsilon_a - \varepsilon_f) = [DNA]/(\varepsilon_b - \varepsilon_f) + 1/K_b(\varepsilon_b - \varepsilon_f) \quad (1)$$

where [DNA] is the concentration of DNA in base pairs, the apparent absorption coefficients ε_a, ε_f, and ε_b correspond to A_{obsd}/[Ru], the extinction coefficient for the free ruthenium complex, and

the extinction coefficient for the ruthenium complex in the fully bound form, respectively. A plot of [DNA]/[ε_a-ε_f] versus [DNA] gave a slope 1/[ε_a-ε_f] and Y intercept equal to 1/K_b[ε_b-ε_f], respectively. The intrinsic binding constant K_b is given by the ratio of the slope to the intercept.

DNA photocleavage assay

The photo-induced DNA cleavage by **Ru1** was examined by gel electrophoresis experiment. Supercoiled pEGFP DNA (0.5 μg) was treated with **Ru1** in 50 mM Tris-HCl solution (pH 7.4), and the samples were then irradiated at room temperature with Xe lamp (450 nm, 150 W). After irradiation, the samples were mixed with sodium dodecyl sulfate (SDS) at a concentration of 1wt%. Afterwards, the samples were incubated at room temperature for 2 h and then was run electrophoretically for 1 h using 1% agarose gel in TBE buffer at 100 V.

Continuous irradiation in presence of CT-DNA.[9]

Continuous irradiation in presence of CT-DNA was performed with a mercury vapour lamp (Osram HBO 200 W) and a 2000 W quartz halogen lamp (Philips), cooled by a system of water circulation. IR (water) and UV (KNO_2) cut-off filters were inserted between the irradiation cell and the exciting source. All the experiments were performed with argon- and air-saturated solutions (2 mL) containing **Ru1** (4 μM) and CT-DNA (40 μM, bases).

Preparation of DNA particles

The DNA particles were prepared by incubating the mixtures containing DNA and **Ru1** at the given +/- ratios in 50 mM Tris-HCl (Tris = Tris(hydroxymethyl)aminomethane) solution (pH 7.4) or in cell culture, followed by vortexing for 30 varied periods to allow equilibration at room temperature.

Gel retardation assay.

Negatively supercoiled pBR322 DNA (7.5 μM) was treated with **Ru1** in 50 mM Tris-HCl solution (pH 7.4), and the solutions were analyzed by electrophoresis for 1.5 h at 75 V on a 1% agarose gel

in TBE buffer (89 mM Tris-borate acid, 2 mM EDTA, pH 8.3). The gel was stained with 1 μg/mL ethidium bromide (EB) and photographed on an Alpha Innotech IS-5500 fluorescence chemiluminescence and visible imaging system.

DNase I protection assay

Ru1-pEGFP DNA particles with 0~2 times DOPE at the +/- ratios of 26.7 containing 1μg pEGFP DNA and 1μg free pEGFP DNA were incubated at 37 °C for 30 min in the presence of 1 unit of DNase-I in the digestion buffer consisting of 50 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$. After DNase-I digestion, the solution was treated with 5 μL of 250 mM EDTA (pH 8.0) for 10 min to inactivate DNase-I and then mixed with sodium dodecyl sulfate (SDS) in 0.1 M NaOH (pH 7.2) at a concentration of 1wt%. Afterwards, the sample was incubated at room temperature for 2 h and then was run electrophoretically for 1 h using 1% agarose gel in TBE buffer at 100 V.

Dynamic light scattering and zeta potential assay.

Dynamic laser light scattering equipment (Brooken Haven BI-200SM) was used to determine the average hydrodynamic diameter and the zeta potential of **Ru1**-pBR 322 DNA particles with 0-2 times DOPE at various +/- ratios in 50 mM Tris-HCl solution (pH 7.4). Typically, 6 runs were measured for each solution, with the average of all the runs reported.

AFM imaging.

The morphology of **Ru1**-pBR 322 DNA particles with 0-2 times DOPE at the +/- ratios of 26.7 was examined with AFM. Samples were placed 10 μL each time and dropped onto a mica substrate, which was freshly cleaved by pulling off the top sheets with tape and 1 min later, the substrate was performed by spin coating (1400 rpm, 30 s) and rinsed with 20 μL of distilled water. AFM images were obtained in air at room temperature with an SPA400 atomic force microscope (AFM) unit and an SPI3800N control station (Seiko Instruments) operated in the tapping mode. Probes made of a single silicon crystal with the cantilever length of 129 mm and the spring constant of 33–62N/m (OMCLAC160TS-W2, Olympus) were used for imaging. The images were

captured in a 256-256 pixels format and analyzed with the software accompanying the imaging module.

Cell viability assay

HeLa cells were maintained in DMEM media with 10% FBS and 1% antibiotic solution at 37 °C at 5% CO_2 in the steri-cycle CO_2 incubator with HEPA Class 100 filters (Thermo Electron Corporation). The cytotoxicity of **Ru1** and **Ru1**-pEGFP DNA particles with 0~2 times DOPE at the +/- ratios of 26.7 was evaluated in HeLa cells by MTT assay.[10] At 3 days after seeding, the cells were counted by hemocytometer and seeded into a 96-well cell-culture plate at a cell density of 1×10^4 cells per well and then incubated for 24 h at 37 °C under 5% CO_2. The complex was then added at indicated concentrations to quadruplicate wells. After 48 h, stock MTT dye solution (20 μL, 5 mg/mL) was added to each well and the microplates were incubated at 37 °C for 4 h. The medium was then removed and buffer (100 μL) containing DMSO (50%) and sodium dodecyl sulfate (20%) was added to the plates and shaken to dissolve the formazan products. A Tecan Infinite M200 monochromator-based multifunction microplate reader was used to measure the optical density of each well with background subtraction at 490 nm. The cell survival rate in the control wells without **Ru1** solutions was considered as 100 % cell survival.

Celluar uptake of DNA particles and Transfection

HeLa cells were grown in DMEM containing 10% FBS at 37 °C in a 5% CO_2 atmosphere. The cells were trypsinized, counted, and adjusted to 1×10^5 cells mL^{-1} and 1mL was added per plate. After 24 hrs, the cell culture medium was replaced with 800 μL serum-free DMEM. **Ru1**-pEGFP DNA particles with 0~2 times DOPE at the +/- ratios of 26.7 containing 1μg pEGFP DNA in 200 μL serum-free DMEM was added to the cells and incubated at 37 °C for 4 h.

For flow cytrometric analysis, after washed with PBS three times, the cells were trypsinized and centrifugated in PBS buffer. Cells were harvested and single cells suspension in 0.5 mL PBS buffer was prepared and subjected to flow cytrometric analysis. A flow cytometer (Coulter Co. USA) was used to measure the fluorescence intensity with excitation at 488 nm.

For TEM imaging, cell processing for TEM analysis was carried out in situ, without

displacement from the culture dish. Cells were fixed in a 0.1 M PBS solution containing 2.5% gluteraldehyde and 4% paraformaldehyde for 1 h. They were then rinsed with 0.1 M PBS and post-fixed in 1% osmium tetroxide solution (extremely toxic; use caution) for 1 h, rinsed with distilled water, stained with 0.5% uranyl acetate for 1 h, dehydrated in a graded series of ethanol (30, 60, 70, 90, and 100%), and embedded in epoxy resin. The resin was polymerized at 60 °C for 48 h. Ultrathin sections (50-75 nm) obtained with an LKB ultramicrotome were stained with 2% aqueous uranyl acetate and 2% aqueous lead citrate and imaged under a 120 kV FEI Tecnai Spirit TEM.

For transfection assay, after washed with PBS three times, the cells were followed by replacement of the medium with fresh DMEM containing 10% FBS, and the cells were incubated for a further 48 h.

Real-time live cell imaging:

Cell nuclei were stained with DAPI (1 μg mL^{-1}) for 20 min. After being washed with PBS twice, cells were cultured in serum-free DMEM for 4 h at 37 °C in a 5% CO_2 atmosphere, then the medium was replaced with fresh DMEM with 10% FBS and incubated for additional 48 h on a thermo-cell culture FCS2 chamber of Carl Zeiss Cell Observer (Jena, Germany). Cell images were captured with a monochromatic CoolSNAP FX camera (Roper Scientific, USA) and analyzed by using AxioVision 4.2 software (Carl Zeiss).

Luciferase assay.

HeLa cells were seeded into a 96-well cell-culture plate at a cell density of 1×10^4 cells per well and then incubated for 24 h at 37 °C under 5% CO_2. Cells were washed with PBS three times and replaced with serum-free DMEM. **Ru1**-plasmid pGL3 control vector DNA particles with 0-2 times DOPE (corresponding to 0.2 μg plasmid/well) were added to each well and the cells were incubated at 37 °C for 4 h. The medium was then replaced with fresh DMEM with 10% FBS and incubated for additional 48 h. The gene activity was determined by comparing average fluorescence intensity of 10,000 cells. Cells were washed with PBS, and harvested and treated for 30 min at 4 °C in end-over-end rotation with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl,

2% Triton X-100, 2% NP40). The luciferase assay was carried out according to the manufacturer's protocol (Promega). Relative light units (RLU) were measured with Varioskan Flash (Thermo Scientific, USA) GloMaxTM 96 microplate luminometer (Promega, USA).

Photostability assay

Before washing with PBS, the HeLa cells were incubated solely with the solution of **Ru1** (10 μM) or DAPI (1 μg mL^{-1}) for 30 min at 37 °C. Then, the experiments were carried out on a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) using a planapochromate 63×/NA 1.4 oil immersion objective. An Ar-Kr laser was served as excitation of the HeLa cells incubated with **Ru1** or DAPI at 405 nm. Emission was collected at 590–630 nm for the HeLa cells incubated solely with **Ru1 (**or at 440–480 nm for DAPI).

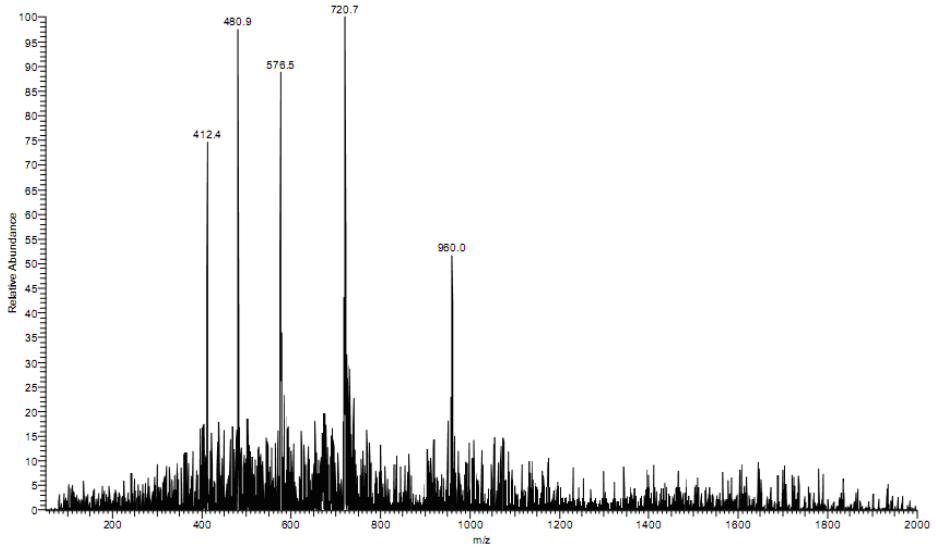


Fig. S1. ESI-MS spectrum of **Ru1** in CH_3OH solution.

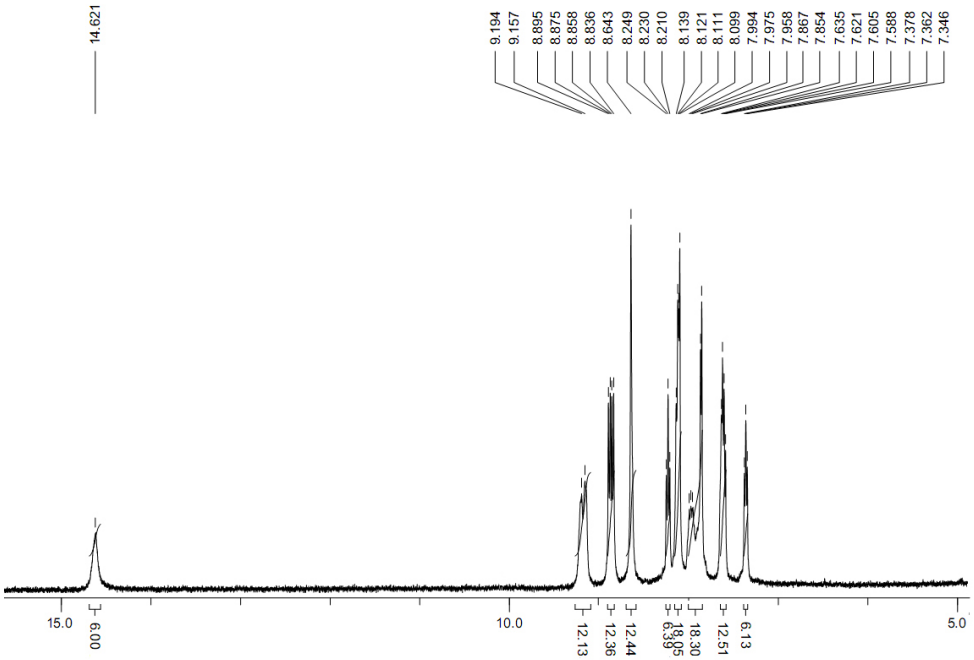


Fig. S2. ^{1}H NMR spectrum of **Ru1** in d_6-DMSO

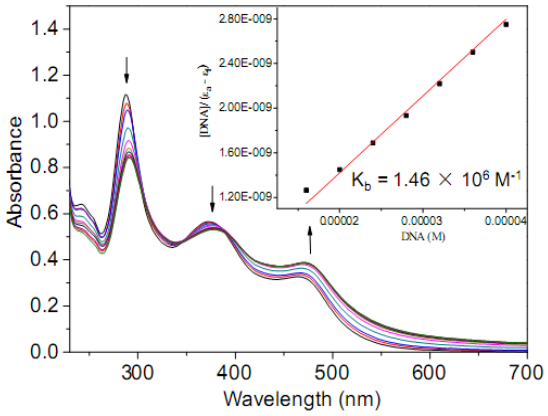


Fig. S3. Changes in absorption spectra of **Ru1** (4 μM) upon addition of CT-DNA (0-40 μM bases). Inset: plot of [DNA]/ (εa - εf) vs [DNA] and the linear fit for the titration of the complex with the DNA.

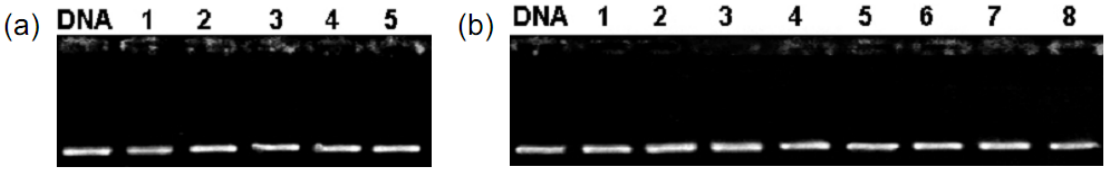


Fig. S4. Agarose gel of the photocleavage of pEGFP DNA (200 ng) and **Ru1** in air. (a) naked DNA as the control, Line 1-5: mixed DNA and **Ru1** at the +/- ratio of (1) 5.3, (2) 13.3, (3) 26.7, (4) 40, (5) 53.3 with 2 h irradiation, respectively. (b) naked DNA as the control, Line 1-8: mixed DNA and **Ru1** with 2 times of DOPE at the +/- ratio of 26.7 with (1) 0.5, (2) 1, (3) 1.5, (4) 2, (5) 2.5, (6) 3, (7) 3.5, (8) 4 h irradiation, respectively. DNA was released by adding 1% SDS to each well.

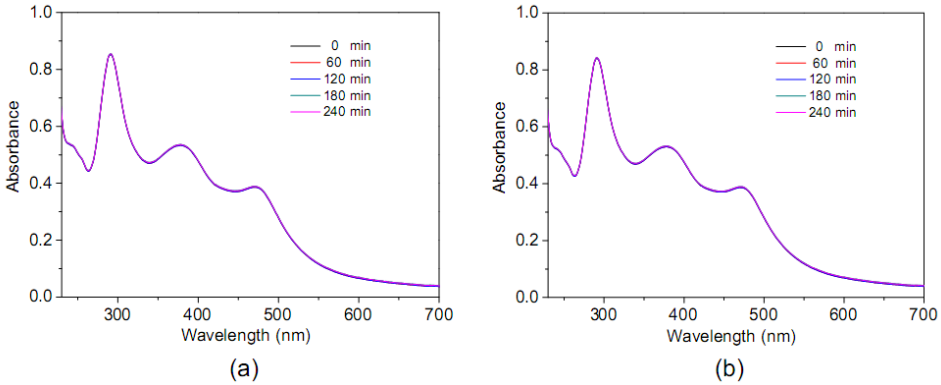
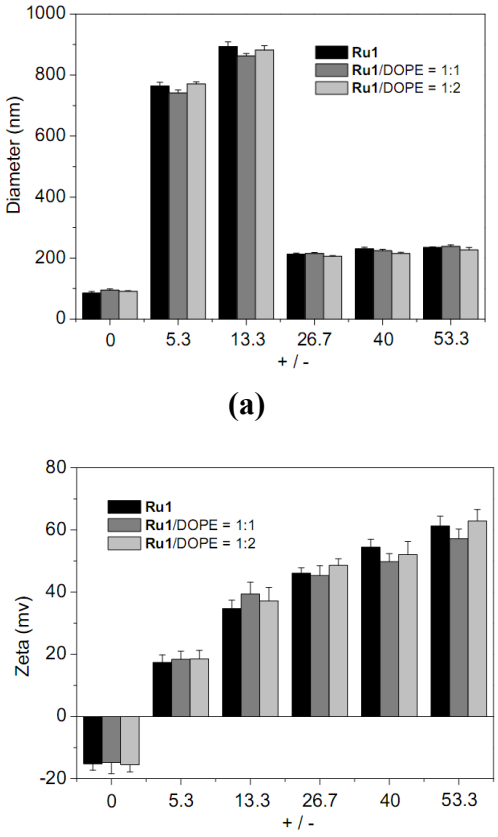


Fig. S5. Changes in the absorption spectra of **Ru1** (4 μM) under visible irradiation in the presence of CT-DNA (40 μM, bases). (a) Air-saturated solution, (b) Argon-saturated solution. Irradiation: time = 0, 60, 120, 180, 240 min.



(a)

(b)

Fig. S6. The diameter (a) and zeta potential (b) of **Ru1**–pBR 322 DNA particles with 0~2 times DOPE at various +/- ratios by DLS. The concentration of pBR 322 DNA was 1.5 μM(bp) in each measurement , respectively.

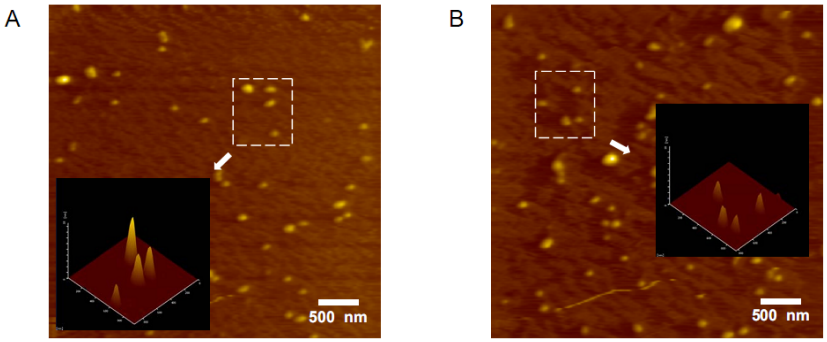


Fig. S7. The diameters of **Ru1**–pBR 322 DNA particles at the +/- ratios of 26.7 measured by AFM. (A) **Ru1**/DOPE = 1:1, (B) **Ru1**/DOPE = 1:2.

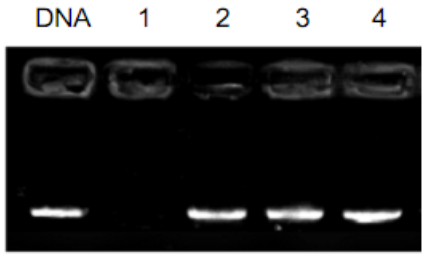


Fig. S8. pEGFP (1 μg) protection from DNase I enzyme by **Ru1** at +/- ratio of 26.7 in the prensence of Dnase I enzyme (Line 1-4). DNA was released by adding 1% SDS to the DNA particles. Lane 1: naked DNA; Line 2 **Ru1** only; Line 3 **Ru1**/DOPE = 1:1; Line 4 **Ru1**/DOPE = 1:2.

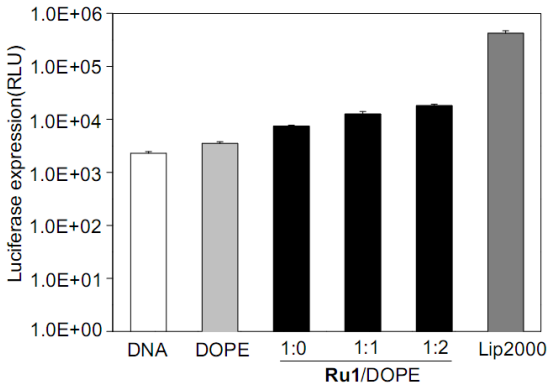


Fig. S9. Transfection efficiencies of **Ru1**-pGL3 DNA particles with 2 times of DOPE at the +/- ratio of 26.7 in HeLa cells by luciferase assays. As controls, DNA, DOPE and lipofectamine 2000 were also investigated.

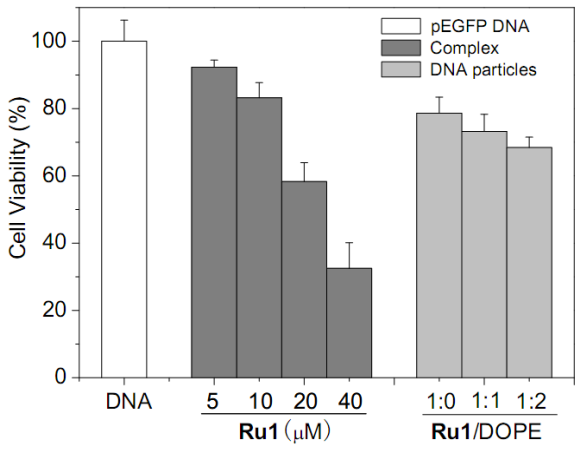


Fig. S10. Cytotoxicity of complex and **Ru1**-pEGFP DNA particles with 0–2 times of DOPE at the +/- ratio of 26.7 in HeLa cells by MTT assays. The concentrations of DNA and **Ru1** were 1.5 μM(bp) and 10 μM per well for the test of DNA particles , and the cytotoxcity of DNA (1.5 μM，bp) was set as the control.

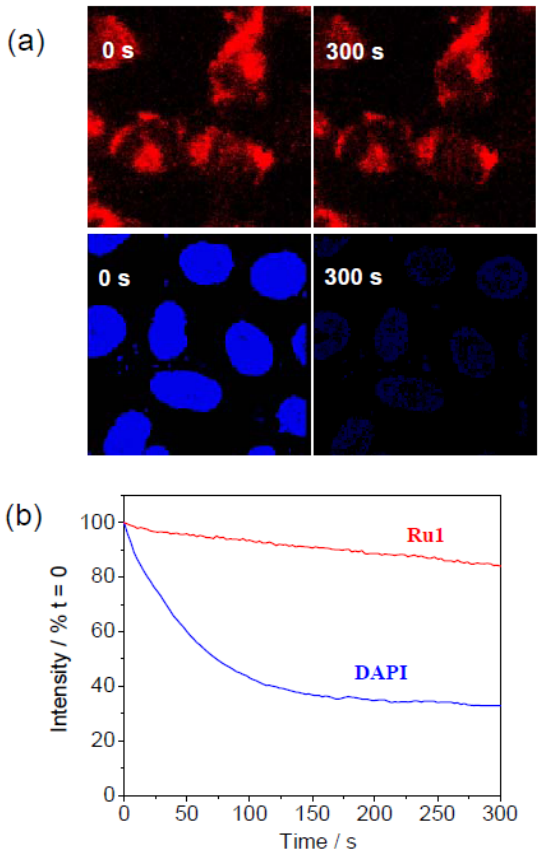


Fig. S11. (a) Comparison of **Ru1** and DAPI for resistance to photo-bleaching. Confocal luminescence images of fixed HeLa cells stained with **Ru1** and DAPI were obtained under the same excitation conditions (405 nm) with different laser scan times (0, 300 s). (b) Luminescence decay curves of **Ru1** and DAPI during the same period.

Reference

1 G. A. Crosby and J. N. Demas, *J. Phy. Chem.*, 1971, **75**, 991.
2 K. Nakamaru, *Bull. Chem. Soc. Jpn.* 1982, **55**, 2697.
3 B. P. Sullivan, D. J. Salmon and T. J. Meyer, *Inorg Chem*., 1978, **17**, 3334.
4 I. P. Evans, A. Spencer and G. Wilkinson, *J. Chem. Soc., Dalton Trans*., 1973, 204.
5 H. Chao, R. H. Li, C. W. Jiang, H. Li, L. N. Ji and X. Y. Li, *J. Chem. Soc., Dalton Trans*., **2001**, 1920.
6 J. Marmur, *J. Mol. Biol.* 1961, **3**, 208.
7 M. E. Reichmann, S. A. Rice, C. A. Thomas and P. Doty, *J. Am. Chem. Soc.*, 1954, **76**, 3047.
8 A. Wolf, G. H. Shimer Jr. and T. Meehan, *Biochemistry*, 1987, **26**, 6392.
9 R. Blasius, H. Nierengarten, M. Luhmer, J.-F. Constant, E. Defrancq, P. Dumy, A. van Dorsselaer, C. Moucheron and A. Kirsch-DeMesmaeker, *Chem. Eur. J.*, 2005, **11**, 1507.
10 M. B. Hansen, S. E. Nielsen and K. Berg, *J. Immunol. Methods*, 1989, **119**, 203.